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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. EV 405 491 038 US

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Zhao-Yi	WANG	Bellevue, Nebraska

Additional inventors are being named on the _____ *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max):

ESTROGEN RECEPTORS AND METHODS FOR THEIR USE

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

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OR

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☑ Application Data Sheet. See 37 CFR 1.76
☐ CD(s), Number of CDs _____
☑ Specification *Number of Pages* 73
☐ Other (specify) _____
☑ Drawing(s) *Number of Sheets* 12

Application Size Fee: If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

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☑ Applicant claims small entity status. See 37 CFR 1.27.
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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.
☐ No.
☑ Yes, the name of the U.S. Government agency and the Government contract number are: CA84328

SIGNATURE David L Provence

Date 13 January 2005

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REGISTRATION NO. 43,022 *(if appropriate)*

TELEPHONE 612-305-1005

Docket Number: 180.0012 0161

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s): Zhao-Yi WANG

Docket No.: 180.0012 0161

Title: ESTROGEN RECEPTORS AND METHODS FOR THEIR USE

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

We are transmitting the following documents along with this Transmittal Sheet (which is submitted in triplicate):

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- X Drawings (12 figures on 12 sheets);
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By: Kelly J. McNulty
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PATENT
Docket No. 180.00120161

ESTROGEN RECEPTORS AND METHODS FOR THEIR USE

Government Funding

The invention described herein was developed with support from the Department of Health and Human Services under Grant Number CA84328. The U.S. Government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Estrogen is a generic term for steroid compounds that are formed in the ovary, the testis, and possibly the adrenal cortex. Examples of estrogens and compounds having estrogen activity include diethylstilbestrol, fosfestrol, hexestrol, polyestradiol phosphate, broparoestrol, chlorotrianisene, dienestrol, diethylstilbestrol, methestrol, colpormon, equilenin, equilin, estradiol, estriol, estrone, ethinyl estradiol, mestranol, mexestrol, quinestradiol and quinestrol. Estrogens regulate diverse physiological processes in reproductive tissues and in mammary, cardiovascular, bone, liver, and brain tissues. Anti-estrogens are used to treat metastatic breast carcinoma and advanced prostate cancer. Estrogens are also used in oral contraceptives. Other uses for estrogens include the relief of the discomforts of menopause, inhibition of lactation, and treatment of osteoporosis, threatened abortion, and various functional ovarian disorders.

The effects of estrogens are mediated via estrogen receptors. The first estrogen receptor (ER) was cloned in 1986 (Green et. al., Nature, 320:134 (1986) and Greene et. al., Science, 231:1150 (1986)). Until 1995, it was assumed that there was only one estrogen receptor responsible for all of the physiological and pharmacological effects of natural and synthetic estrogens and antiestrogens. However, in 1995, a second estrogen receptor was cloned (Kuiper et. al., PNAS, 93:5925 (1996)). The first estrogen receptor discovered is now called estrogen receptor-alpha (ER-α) and the second estrogen receptor is called estrogen receptor-beta (ER-β).

ER-α and ER-β share a common structural architecture (Zhang et. al., FEBS Letters, 546:17 (2003) and Kong et. al., Biochem. Soc. Trans., 31:56 (2003)). Both are composed of three independent but interacting functional domains: the N-terminal A/B domain, the C or DNA-binding domain, and the D/E/F or ligand-binding domain (Figure 1). The N-terminal domain of ER-α encodes a ligand-independent activation function (AF-1), a region involved in interaction with co-activators, and transcriptional activation of target genes. The DNA-binding domain or C domain contains a two zinc-finger structure, which plays an important role in receptor dimerization and binding to specific DNA sequences. The C-terminal D/E/F domain is a ligand-binding domain that mediates ligand binding, receptor dimerization, nuclear translocation, and a ligand-dependent transactivation function (AF-2). The relative contributions that both AF-1 and AF-2 exert on transcriptional control vary in a cell-specific and DNA promoter-specific manner (Berry et. al., EMBO J., 9:2811 (1990) and Tzukerman et. al., Mol. Endocrin., 8:21 (1994)).

A 46-kDa ER-α isoform lacking the first 173 amino acids of the full-length gene product of the ER-α gene (A/B or AF-1 domain) was shown to be derived from alternative splicing of the ER-α gene by skipping exon 1 (Flouriot et. al., EMBO J., 19:4688 (2000)). This alternative splicing event generates an mRNA that has an AUG in a favorable Kozak sequence for translation initiation in frame with the remainder of the original open reading frame. Therefore, this new isoform of ER-α was named as ER-α46 and the original one was named ER-α66 (Flouriot et. al., EMBO J., 19:4688 (2000)). ER-α46 forms homodimers and binds to an estrogen response element (ERE), and it can also form heterodimers with ER-α66 (Flouriot et. al., EMBO J., 19:4688 (2000)). ER-α46 homodimers show a higher affinity for an ERE than ER-α66 homodimers. Furthermore, the ER-α46/66 heterodimers form preferentially over the ER-α66 homodimers and ER-α46 acts competitively to inhibit transactivation mediated by the AF-1 domain of liganded-ER-α66, but does not effect AF-2-dependent transactivation (Floutiot et. al., EMBO J., 19:4688 (2000)). Therefore, it is thought that ER-α46 is a naturally occurring isoform of ER-α that regulates estrogen signaling mediated by the AF-1 domain of ER-α66.

ER-α is expressed in approximately 15-30% of luminal epithelial cells and not at all in any of the other cell types in the normal human breast. Dual label immunofluorescent techniques revealed that ER-α-expressing cells are separate from those labeled with proliferation markers in both normal human and rodent mammary glands (Clarke et. al., Cancer Res., 57:4987 (1997)). ER-α expression is increased at the very earliest stages of ductal hyperplasia and increases even more with increasing atypia, such that most cells in atypical ductal hyperplasias and in ductal cancer in situ of low and intermediate nuclear grade contain the ER-α (Khan et. al., Cancer Res., 54:993 (1994) and Lawson et. al., Lancet, 351:1787 (1994)). As ER-α expression increases, the inverse relationship between receptor expression and cell proliferation become dysregulated (Shoker et. al., Amer. Jour. Path., 155:1811 (1999)). Approximately 70% of invasive breast carcinomas express the ER-α and most of these tumors contain ER-α-positive proliferating cells (Clarke et. al., Cancer Res., 57:4987 (1997)).

Estrogen receptors are members of the nuclear receptor superfamily of ligand-activated transcription factors that control numerous physiological processes. This control often occurs through the regulation of gene transcription (Katzenellenbogen and Katzenellenbogen, Breast Cancer Res., 2:335 (2000); Hull et al., J. Biol. Chem., 276:36869 (2001); McDonnell and Norris, Science, 296:1642 (2002)). The estrogen receptor utilizes multiple mechanisms to either activate or repress transcription of its target genes. These mechanisms include: (a) direct interaction of the ligand-occupied receptor with DNA at estrogen response elements followed by recruitment of transcriptional coregulator or mediator complexes, (b) interaction of the ligand-occupied ER with other transcription factors such as AP-1 (Kushner at al., J. Steroid Biochem. Mol. Biol., 74:311 (2000)), Sp1 (Safe, Vitam. Horm., 62:231 (2001)) or NF-κB (McKay and Cidlowski, Endocr. Rev., 20:435 (1999)), or (c) indirect modulation of gene transcription via sequestration of general/common transcriptional components (Harnish et al., Endocrinology, 141:3403 (2000) and Speir et al., Circ. Res., 87:1006 (2000)). In addition, the ability of an estrogen receptor to regulate transcription through these various mechanisms appears to be cell-type specific, perhaps due to differences in the complement of transcriptional coregulatory factors

available in each cell type (Cerillo et al., J. Steroid Biochem. Mol. Biol., 67:79 (1998); Evans et al., Circ. Res., 89:823 (2001); Maret et al., Endocrinology, 140:2876 (1999)). Also, transcriptional regulation is dependent upon the nature of the ligand, with various natural and synthetic selective estrogen receptor modulators acting as either estrogen receptor agonists or antagonists through each of these various mechanisms (Shang and Brown, Science, 295:2465 (2002); Katzenellenbogen and Katzenellenbogen, Science, 295:2380 (2002); Margeat et al., J. Mol. Biol., 326:77 (2003); Dang et al., J. Biol. Chem., 278:962 (2003)).

Another signaling pathway mediated by estrogens, also known as a 'non-classic', 'non-genomic' or 'membrane signaling' pathway, exists that involves cytoplasmic proteins, growth factors and other membrane-initiated signaling pathways (Segars et. al., Trends Endocrin. Met., 13:349 (2002)). Several intracellular signaling pathways have been shown to cross-talk with rapid estrogen-initiated effects: the adenylate cyclase pathway (Aronica et. al., PNAS, 91:8517 (1994)), the phospholipase C pathway (Le Mellay et. al., J. Cell. Biochem., 75:138 (1999)), the G-protein-coupled receptor-activated pathways (Razandi et. al., Mol. Endocrin., 13:307 (1999)) and the mitogen activated protein kinaase (MAPK) pathway (Watters et. al., Endocrinology, 138:4030 (1997)). However, all membrane forms described to date are related to ER-α but not ER-β (Segars et. al., Trends Endocrin. Met., 13:349 (2002)).

Estrogen signaling has been associated pathologically with an increased risk for breast and endometrial cancer (Summer and Fuqua, Semin. Cancer Biol., 11:339 (2001); Turner et al., Endocr. Rev., 15:275 (1994); Farhat et al., FASEB J., 10:615 (1996); Beato et al., Cell, 83:851 (1995); Dobrzycka et al., Endo. Rel. Cancer, 10:517 (2003)). Consequently, estrogen receptors have been found to be essential in the initiation and development of most of these cancers. Current endocrine therapies for estrogen receptor-positive breast cancers are primarily designed to target estrogen levels, estrogen receptor levels, or the activity of estrogen and estrogen receptors. Use of a partial antiestrogen, tamoxifen, in the management of early-stage breast cancer has clearly demonstrated an increase in both disease-free and overall survival. In addition, recent studies demonstrate that tamoxifen can be used as a chemopreventive agent for hormone-dependent breast cancer. The major concerns of long-term therapy

with tamoxifen are its uterotropic effects, which result in an increase risk for endometrial cancer, and the acquired clinical resistance to tamoxifen. This has led to the active pursuit of better selective estrogen receptor modulators (SERM) that display the optimal agonistic or antagonistic activities in various estrogen responsive target tissues.

Accordingly, what are needed are additional methods and materials that can be used to screen for agents that modulate estrogen signaling, as well as methods and materials that can be used to modulate estrogen signaling.

SUMMARY OF THE INVENTION

The invention provides polypeptides including estrogen receptors, estrogen receptor isoforms, estrogen receptor protein fragments, fusion polypeptides, polyproteins and analogs thereof. The polypeptides may be biologically active. The invention also provides peptidomimetics of the polypeptides of the invention. The peptidomimetics may have one or more amide bonds replaced with a non-amide bond linkage. Preferably the petidomimetics are biologically active.

Polynucleotides that encode the polypeptides of the invention are also provided. In a preferred embodiment, these polynucleotides are inserted into, and form part of, a vector. The polynucleotides of the invention can be inserted into an expression cassette or an expression vector. The polynucleotides can be ribonucleic acid or deoxyribonucleic acid. These polynucleotides can be made according to methods provided by the invention.

The invention provides antibodies and peptide aptamers that bind to a polypeptide or peptidomimetic as described herein. In some aspects, the antibody or peptide aptamer does not bind to an ER-α46 or ER-α66 estrogen receptor isoform. In other aspects, the antibody or peptide aptamer only binds to an ER-α36 estrogen receptor isoform. The antibody can be a polyclonal antibody or a monoclonal antibody.

The invention provides a composition containing a polypeptide, peptidomimetic, antibody, or peptide aptamer as described herein. The composition may additionally contain a pharmaceutically acceptable carrier. In a preferred embodiment, the composition is formulated for transdermal administration, oral

administration, intravenous administration, intraocular administration, intranasal administration, inhalation administration, parenteral administration, and/or rectal administration.

The invention provides a method to determine if a test cell responds to, or is refractory to, treatment with a therapeutic agent that involves determining the ratio of ER-α36 to ER-α66, ER-α46 or ER-β in the test cell, and comparing the ratio determined in the test cell to a ratio of ER-α36 to ER-α66, ER-α46 or ER-β in a control cell. The level of ER-α36 in a sample or cell may also be quantitated. Preferably the control cell is not refractory to treatment with a therapeutic agent. More preferably, the control cell is refractory to treatment with the therapeutic agent. Preferably the therapeutic agent is an estrogen receptor antagonist or a selective estrogen receptor modulator. More preferably the therapeutic agent is centchroman, delmadinone acetate, droloxifene, idoxifene, tamoxifen, raloxifene, toremifene, a bisphosphonate, calcitonin, tribolone, parathyroid hormone, strontium ranelate, a growth factor, or a cytokine. Most preferably the therapeutic agent is tamoxifen.

A method to increase or decrease expression of an estrogen regulated gene in a cell is provided by the invention. The method involves contacting a cell having an estrogen receptor with a polypeptide, a peptidomimetic, a peptide aptamer, an antibody, a composition, or any combination thereof of the invention. The expression of an estrogen regulated gene can be increased or decreased. The cell can be isolated or contained within the body of a mammal.

The invention further provides a method to reduce or ameliorate an estrogen related disorder in a mammal. The method involves administering an effective amount of a polypeptide, a peptidomimetic, an antibody, a peptide aptamer, a composition, or any combination thereof as described herein to the mammal in need of such treatment. Compositions may include one or more SERMs, estrogen, or anti-estrogens. The method can be used to increase or decrease signaling by the estrogen receptor. Preferably the estrogen related disorder is breast cancer, prostate cancer, Alzheimer's disease, Parkinson's disease, multiple sclerosis, depression, bipolar disorder, schizophrenia, osteoporosis, or ischemic stroke.

A method to reduce estrogen receptor promoted proliferation of a cell is provided by the invention. The method involves contacting a cell with a polypeptide, a peptidomimetic, or a composition of the invention such that estrogen receptor signaling is reduced or increased, preferably reduced, more preferably, eliminated. Preferably the cell is a uterine cell. More preferably the cell is a breast cancer cell. Preferably the cell is contained within a mammal. More preferably the cell is contained within a human.

The invention provides a method to increase estrogen receptor activity in a cell. The method involves contacting a cell with an antibody, a peptide aptamer, a composition containing a peptide or peptide aptamer of the invention, or any combination thereof. Compositions may include one or more SERMs, estrogen, or anti-estrogens. The cell may be an isolated cell. Preferably the cell is contained within a mammal. More preferably the cell is contained within a human. The method may be used to reduce or ameliorate Alzheimer's disease, Parkinson's disease, multiple sclerosis, depression, bipolar disorder, schizophrenia, osteoporosis, or ischemic stroke.

A method to screen for an agent that promotes or inhibits dimerization of ER-α36 with ER-α36, ER-α46, ER-α66, or ER-β is provided by the invention. The method involves determining if a candidate agent increases or decreases formation of a complex that includes ER-α36 and ER-α46, ER-α36 and ER-α66, or ER-α36 and ER-β, in a cell that was contacted with a candidate agent compared to formation of a corresponding complex in a cell that was not contacted with the candidate agent. The complex can be isolated from the cell. Preferably an antibody or peptide aptamer of the invention is used to isolate the complex.

The invention provides an ER-α36 promoter that provides for ER-α36 regulated expression of an operably linked open reading frame. The ER-α36 promoter can be inserted into a vector. In one aspect, the ER-α36 promoter is operably linked to an open reading frame. The open reading frame can encode a detectable marker. The detectable marker can be a polypeptide. The detectable marker can be an antigenic polypeptide, an enzyme, a toxin, or a drug resistance polypeptide. The detectable marker can also be a fluorescent polypeptide.

A method to identify an agent that increases or decreases ER-α36 promoter driven expression of an open reading frame is provided by the invention. The method involves comparing expression of a detectable marker encoded by an open reading frame that is operably linked to an ER-α36 promoter in a test cell that was contacted with a candidate agent, to expression of a detectable marker encoded by an open reading frame that is operably linked to an ER-α36 promoter in a control cell that was not contacted with a candidate agent.

The invention provides a method to screen for a ligand that is bound by an ER-α36 estrogen receptor complex. Generally, the method involves contacting a cell that includes a reporter construct and that expresses an ER-α36-fusion polypeptide with a candidate ligand, and determining if the candidate ligand increases or decreases expression of a detectable marker when compared to expression of a corresponding control cell that was not contacted with the candidate ligand.

Kits are also provided by the invention. A kit can contain packaging material and an antibody or peptide aptamer that specifically binds to an ER-α36 isoform of an estrogen receptor. Preferably a kit contains packaging material and a first antibody that specifically binds to an ER-α36 isoform of an estrogen receptor, and a second antibody that specifically binds to an ER-α46 isoform of an estrogen receptor. More preferably a kit contains packaging material and a first antibody that specifically binds to an ER-α36 isoform of an estrogen receptor, and a second antibody that specifically binds to an ER-α66 isoform of an estrogen receptor. Preferably the first antibody or the second antibody is coupled to a detectable marker. More preferably the first antibody and the second antibody are each bound to a detectable marker.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the domain structure representation of Human estrogen receptor-alpha (ER-α) isoforms. Domains (labeled A-F), amino acid sequence numbering, AF-1 and AF-2, the DNA binding domain, the ligand binding domain, and the dimerization domain are shown. The phosphorylation sites and function of each domain are also indicated.

Figure 2 is a schematic demonstrating the possible crosstalk between the membrane and genomic signaling pathways of ER-α. Cav-1 represents caveolin-1, ER-α represents estrogen receptor-alpha, RTK represents a receptor tyrosine kinase, Ras represents the Ras oncogene, Mek represents the Mek oncogene, MAPK represents a mitogen activated protein kinase, PI3K represents phosphoinositol-triphosphate kinase, AKT represents protein kinease 13, PDK1 represents phosphoinositol-dependent protein kinase and RSK represents p90 ribosome S6 kinase.

Figure 3 is a picture showing that pRET-infected MCF10A cells grow a big colony in soft-agar in the presence of estradiol (E2). The ST1 clone shows an accelerated growth in E2-containging soft–agar. MCF7 and MCF10A cells are included as a positive and negative control, receptively.

Figure 4 is a western blot showing downregulation of Caveolin-1 (Cav-1) expression in pRET-infected MCF10A cells. Equal amounts of total cellular extracts from various cell lines were analyzed by western blot using a rabbit anti-Cav-1 antibody (N20). The position of Cav-1 is indicated by an arrow and the cell extract analyzed in each lane is indicted above each lane.

Figure 5 is a western blot showing upregulation of ER-α expression in pRET-infected MCF10A cells. Equal amounts of total cellular extracts from various cell lines were analyzed by western blot using antibodies against ER-α (H222) and ER-β. The positions of ER-α and ER-β are indicated by arrows and the cell extract analyzed in each lane is indicted above each lane.

Figure 6 is a western blot showing activation of ERK phosphorylation in pRET-infected MCF10A cells. Equal amounts of total cellular extracts from the cell lines were analyzed by western blot using antibodies against ERK and phosphorylated ERK.

Figure 7 is a western blot showing the existence of three ER-α proteins in Cav-1 haploinsufficient cells, ST1and ST3, and MCF7 breast cancer cells. Equal amounts of total cellular extracts from the cell lines were analyzed by Western blot using the H222 antibody against ER-α. The positions of ER-α66, ER-α46 and ER-

α36 are indicated by arrows and the cell extract analyzed in each lane is indicted above each lane.

Figure 8 illustrates the genomic organization of the human ER-α gene. The location of multiple promoters are shown as arrows. The translation start and stop sites are indicated as AUG and UGA. The exons are shown as numbered boxes. Intron 1 is also shown with the exon 1' in a box. The lower panel shows the mRNA structure of ER-α isoforms. Poly A sites are indicated by AAA.

Figure 9 is a picture of an agarose gel showing the isolation of cDNA encoding the open-reading frame of ER-α36 by PCR. The position of the cDNA in the gel is indicated by an arrow.

Figure 10 shows the predicted amino acid sequence of the ER-α36 open-reading frame. The amino acid positions are indicated by numbers on the left side of the amino acid sequence (SEQ ID NO: 20).

Figure 11 shows a western blot analysis of ER-α66, ER-α46 and ER-α36. The lanes marked ER-α66, ER-α46 and ER-α36 represent separated cultures of HEK 293 cells that were transfected with expression plasmids encoding the indicated estrogen receptor isoform, and lysed two days after being transfected. The lysate of each transfectant was immunodetected with an anti-ER-α antibody (H222). The cell extracts from MCF7 cells are used as a positive control. The positions of ER-α66, ER-α46 and ER-α36 are indicated by arrows.

Figure 12 shows the DNA sequence of the 5' flanking sequence of the gene that encodes ER-α36 and which includes the ER-α36 promoter. The putative transcription binding sites are underlined and the proteins that bind to the nucleic acid sequence are also indicated. The initiation site of the cDNA is also indicated by an arrow (SEQ ID NO: 22).

DETAILED DESCRIPTION

It has been discovered that downregulation of the Caveolin-1 (Cav-1) system constitutively activates the mitogen activated protein kinase (MAPK) pathway, activates expression of estrogen receptor-alpha (ER-α), and triggers positive estrogen signaling. This discovery has, for the first time, provided a clear link between

activated MAPK signaling and mammary tumorigenesis, especially breast cancer progression that is stimulated by estrogens. This discovery strongly suggests that Cav-1 plays an important role in maintaining normal growth of mammary epithelial cells by coordinating the cross-talk between the MAPK and estrogen signaling pathways, and its downregulation may contribute to dysregulation of these two important pathways which eventually lead to mammary tumorigenesis. A schematic of the estrogen signaling pathway and the MAPK signaling pathway is presented in Figure 2.

An estrogen receptor-alpha isoform has also been identified and cloned. This 36-kDa isoform (ER-α36) of estrogen receptor-alpha is generated from a promoter located in the first intron of the original 66-kDa ER-α (ER-α66) gene. ER-α36 differs from ER-α66 because it lacks both transcriptional activation domains (AF-1 and AF-2) but retains the DNA-binding, dimerization and most of the ligand-binding domains. The structure of ER-α36 indicates that ER-α36 is an important regulator of estrogen signaling. ER-α36 may also mediate the membrane effects of estrogen signaling as it is thought to be expressed in the plasma membrane, cytosol, and nucleus.

ER-β has been proposed as a constitutive regulator of ER-α66 mediated estrogen signaling. The finding that ER-α46 lacking the AF-1 domain can dimerize to ER-α66 and inhibit the transactivation activity mediated by the AF-1 domain of ER-α66 indicates that ER-α46 plays a regulatory role in the functional activity mediated by the AF-1 domain of ER-α66. ER-α36 lacks both AF-1 and AF-2 domains. Thus, it is thought that ER-α36 inhibits biological functions mediated by both AF-1 and AF-2 of ER-α66, and AF-2 mediated functions of ER-α46 as well. With regulation mediated by ER-α36 and ER-α46, both of which might be expressed at different levels in different tissues, ER-α66 may function differently in different target tissues. Such a mechanism is thought to provide an explanation for the pleiotrophic roles of estrogen signaling in different biological processes.

Polypeptides and peptidomimetics of the Invention

The invention provides polypeptides. As used herein, the term "polypeptide" refers broadly to a polymer of two or more amino acids joined together by peptide bonds. The terms peptide, oligopeptide, and protein are all included within the definition of polypeptide and these terms are used interchangeably. It should be understood that these terms do not connote a specific length of a polymer of amino acids, nor are they intended to imply or distinguish whether the polypeptide is produced using recombinant techniques, chemical or enzymatic synthesis, or is naturally occurring. Numerous examples of polypeptides that are within the scope of the invention are disclosed and described herein. Examples of polypeptides of the invention include estrogen receptors, estrogen receptor isoforms, estrogen receptor fragments, fusion polypeptides, polyproteins, analogs and peptidomimetics thereof.

The polypeptides of the present invention may be biologically active. Examples of polypeptides that are biologically active include polypeptides of the invention that are able to increase or decrease expression of an estrogen regulated gene. Another example of a bioassay that can be used to determine if a polypeptide of the invention is biologically active involves contacting a cell that expresses an estrogen receptor with the polypeptide and determining if translocation of estrogen receptors to the nucleus of the cell is increased or decreased in the presence of estrogen, when compared to nuclear translocation in a control cell that was not contacted with the polypeptide of the invention.

The invention provides a polypeptide having the amino acid sequence depicted in SEQ ID NO:20. This polypeptide, and related polypeptides as described herein, are also referred to herein as ER-α36, ER-α36 isoform, and ER receptor α36-subunit. As shown in Figure 1, the ER-α36 isoform lacks amino-terminal amino acid residues 1-183, carboxyl-terminal amino acid residues 430-595, and has an addition of 27 amino acid residues to its C-terminus when compared to the ER-α66 isoform. Estrogen receptor alpha isomers include ER-α36, ER-α46, ER-α66. Estrogen receptor beta isomers include ER-β. The present invention also provides estrogen receptors that include an ER-α36 isoform. Without intending to be limiting, the ER-α36 isoform is

believed to modulate the response of a cell to estrogen through regulation of estrogen receptor function by forming a dimer with ER-α66, ER-α46 or ER-β. Further, ER-α36 is thought to lack activation factor 1 (AF-1) and activation factor 2 (AF-2) activity. However, ER-α36 is thought to retain an intact dimerization domain that allows ER-α36 to dimerize with an ER-α46, ER-α66 or ER-β. This interaction is thought to allow ER-α36 to modulate the activity of ER-α46, ER-α66 and ER-β containing estrogen receptors.

Polypeptides of the present invention includes polypeptides having an amino acid sequence that is at least 70% identical to SEQ ID NO:20. Such polypeptides include those having an amino acid sequence that is at least single unit percentages greater than 70% identical to SEQ ID NO:20, for example 71%, 72%, 73% identity, and so on to 100% identity to SEQ ID NO:20. Preferably, the polypeptide includes those having an amino acid sequence that is, in increasing order of preference, at least about 80% identity, at least about 90% identity, or at least about 95% identity to SEQ ID NO:20. Most preferably, the estrogen receptor isoform includes a polypeptide that has 100% identity to SEQ ID NO:20. Preferably the polypeptides are biologically active.

Percent identity between two polypeptide sequences is generally determined by aligning the residues of the two amino acid sequences to optimize the number of identical amino acids along the lengths of their sequences; gaps in either or both sequences are permitted in making the alignment in order to optimize the number of identical amino acids, although the amino acids in each sequence must nonetheless remain in their proper order. Preferably, two amino acid sequences are compared using the Blastp program, version 2.0.9, of the BLAST 2 search algorithm, as described by Tatusova et al. (FEMS Microbiol. Lett., 174, 247-250 (1999)), and available on the world wide web at www.ncbi.nlm.nih.gov/gorf/bl2.html. Preferably, the default values for all BLAST 2 search parameters are used, including matrix = BLOSUM62; open gap penalty = 11, extension gap penalty = 1, gap x_dropoff = 50, expect = 10, wordsize = 3, and optionally, filter on. In the comparison of two amino acid sequences using the BLAST search algorithm, structural similarity is referred to as "identity."

Polypeptides that are fragments of the ER-α36 estrogen receptor isoform are also provided by the invention. Such a fragment is exemplified by a fragment having the amino acid sequence GISHVEAKKRILNLHPKIFGNKWFPRV (SEQ ID NO:1). Such fragments are useful for preparing antibodies and peptide aptamers that specifically bind to the ER-α36 estrogen receptor isoform, and which can be used to determine if a cell is refractory to, for instance, tamoxifen treatment. Such fragments are also thought to be useful for regulating an estrogen receptor by interacting with the ER-α46 estrogen receptor isoform, or the ER-α66 estrogen receptor isoform. These fragments can be identified by expressing a polynucleotide sequence encoding the fragment in a cell that expresses an estrogen receptor, and determining if expression of the fragment alters the response of the cell to estrogen. For example, alteration of the cellular response to estrogen can be determined through use of a gene chip to identify if a fragment causes increased or decreased expression of a gene. Such methods are known in the art. Examples of fragments include an estrogen receptor isoform that has been truncated at either the N-terminus, or the C-terminus, or both, by one or more amino acids, as long as the fragment contains at least 5 amino acids, more preferably at least 7 amino acids, even more preferably at least 10 amino acids, and most preferably at least 12 amino acids.

The invention provides fusion polypeptides having a carrier polypeptide coupled to a polypeptide of the invention. A carrier polypeptide may be used to increase or decrease the solubility of a fusion polypeptide. The carrier polypeptide may also be used to increase the immunogenicity of the fusion polypeptide to increase production of antibodies that bind to a polypeptide of the invention. For example, a carrier polypeptide may be fused to a fragment of a polypeptide having SEQ ID NO:20 to facilitate production of antibodies that recognize and bind ER-α36. Such a fragment is exemplified by a polypeptide having SEQ ID NO:1 or fragments thereof. The invention is not limited by the types of carrier polypeptides used to create fusion polypeptides of the invention. Examples of carrier polypeptides include keyhole limpet hemacyanin, bovine serum albumin, ovalbumin, mouse serum albumin, rabbit serum albumin, and the like. The carrier polypeptides may also be used to provide for the separation or detection of a fusion polypeptide. Accordingly, a fusion polypeptide

can be detected or isolated by interaction with other components that bind to the carrier polypeptide portion of the fusion polypeptide. For example, a fusion polypeptide having avidin as a carrier polypeptide can be detected or separated with biotin through use of known methods. A carrier polypeptide may also be used to cause the fusion polypeptide to form an inclusion body upon expression within a cell. A carrier polypeptide can also be an export signal that causes export of a fusion polypeptide out of a cell, or directs a fusion polypeptide to a compartment within a cell, such as the periplasm.

For example, an expression cassette can be designed to express a polyprotein that includes biotin coupled to ten copies of a polypeptide of the invention that are connected to each other by a chemical or protease cleavable linker. The polyprotein can be expressed within a cell and then bound to an avidin support such that the polyprotein is immobilized. Cellular contaminants can then be washed away to allow isolation of the polyprotein. The polyprotein can then be cleaved to release polypeptides of the invention. These polypeptides can be purified through use of numerous art recognized methods, such as gel filtration chromatography, ion exchange chromatography, and the like.

A carrier polypeptide may be coupled to polypeptide of the invention through use of routine recombinant methods. A carrier polypeptide may also be coupled to a polypeptide of the invention through use of chemical linking methods, or through use of a chemical linker. Such coupling methods are known in the art and have been described. Harlow et al., Antibodies: A Laboratory Manual, page 319 (Cold Spring Harbor Pub. 1988); Taylor, Protein Immobilization, Marcel Dekker, Inc., New York, (1991).

The invention also provides polyproteins. Generally, polyproteins include two or more polypeptides of the invention that are continuously linked into a single amino acid chain. The polypeptides can be connected by linkers (see Stahl et al., U.S. Patent No. 6,558,924). Such a polyprotein can be isolated and then cleaved to produce polypeptides or coupled polypeptides of the invention. The polyprotein can be cleaved through use of numerous methods, such as chemical or protease cleavage. Accordingly, linkers can be designed to be cleaved by specific proteases or chemicals. Examples of chemicals that can be used to cleave polyproteins of the invention

include cyanogen bromide (-Met↓-), formic acid (70%) and heat (-Asp↓Pro-), hydroxylamine at pH 9 and heat (Asn↓Gly-), iodosobenzoic acid-2-(2-nitrophenyl)-3-methyl-3-bromoindole-nine in 50% acetic acid (-Trp↓), and the like. Examples of enzymes that can be used to cleave polyproteins of the invention include Ala-64 subtilisin (-Gly-Ala-His-Arg↓), clostripain (-Arg↓ and Lys-Arg↓), collagenase (-Pro-Val↓Gly-Pro-), enterokinase (-Asp-Asp-Asp-Asp-Lys↓), factor Xa (-Ile-Glu (or Asp)-Gly-Arg↓), renin (-Pro-Phe-His-Leu↓Leu-), α-thrombin (-Leu-Val-Pro-Arg↓Gly-Ser-), trypsin (-Arg↓ or -Lys↓), chymotrypsin, tobacco etch virus protease (-Glu-Asn-Leu-Tyr-Phe-Gln↓Gly-), and the like. Polyproteins may be used to increase the production efficiency of the polypeptides of the invention. Polypeptides are often times difficult to produce due to proteolytic susceptibility and inefficient expression. These difficulties may be overcome through use of polyproteins due to increased protease resistance and expression. Methods to produce polyproteins are known in the art (see Coolidge et al., U.S. Patent No. 6,127,150).

The polypeptides of the invention include analogs that have been modified by the addition, substitution, or deletion of one or more contiguous or noncontiguous amino acids, or that have been chemically or enzymatically modified, e.g., by attachment of a reporter group, by an N-terminal, C-terminal or other functional group modification or derivatization, or by cyclization, as long as the analog retains biological activity or is able to stimulate the production of antibodies that bind to ER-α36. An analog can thus include additional amino acids at one or both of the termini of a polypeptide. In some aspects, the invention provides polypeptides that are not analogs.

Substitutes for an amino acid in the polypeptides of the invention are preferably conservative substitutions, which are selected from other members of the class to which the amino acid belongs. For example, it is well-known in the art of protein biochemistry that an amino acid belonging to a grouping of amino acids having a particular size or characteristic (such as charge, hydrophobicity and hydrophilicity) can generally be substituted for another amino acid without substantially altering the structure of a polypeptide. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and tyrosine. Polar neutral amino acids include glycine,

serine, threonine, cysteine, tyrosine, asparagine and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Examples of preferred conservative substitutions include Lys for Arg and vice versa to maintain a positive charge; Glu for Asp and vice versa to maintain a negative charge; Ser for Thr so that a free -OH is maintained; and Gln for Asn to maintain a free NH_2. Related amino acids such as 3-hydroxyproline, 4-hydroxyproline, homocysteine, 2-aminoadipic acid, 2-aminopimelic acid, γ-carboxyglutamic acid, β-carboxyaspartic acid, and the corresponding amino acid amides; ornithine, homoarginine, N-methyl lysine, dimethyl lysine, trimethyl lysine, 2,3-diaminopropionic acid, 2,4-diaminobutyric acid, homoarginine, sarcosine and hydroxylysine; substituted phenylalanines, norleucine, norvaline, 2-aminooctanoic acid, 2-aminoheptanoic acid, statine and β-valine; and naphthylalanines, substituted phenylalanines, tetrahydroisoquinoline-3-carboxylic acid, and halogenated tyrosines may be exchanged for a like amino acid.

The invention provides peptidomimetics of the polypeptides of the invention. A peptidomimetic describes a polypeptide in which at least one of the peptide bonds has been replaced with a non-peptide bond, such as those commonly used in the pharmaceutical industry as non-peptide drugs, with properties analogous to those of the template polypeptide. (Fauchere, J., Adv. Drug Res., 15: 29 (1986), Evans et al., J. Med. Chem., 30:1229 (1987), and Janda et al., U.S. Patent No. 6,664,372). Peptidomimetics are structurally similar to polypeptides having peptide bonds, but have one or more peptide linkages optionally replaced by a linkage such as, --CH_2NH--, --CH_2S--, --CH_2--CH_2--, --CH=CH-- (cis and trans), --$COCH_2$--, --$CH(OH)CH_2$--, and --CH_2SO--, by methods known in the art. Advantages of peptidomimetics over natural polypeptide embodiments may include more economical production, greater chemical stability, altered specificity and enhanced pharmacological properties such as half-life, absorption, potency and efficacy.

Substitution of one or more amino acids within a polypeptide or a peptidomimetic with a D-amino acid of the same type (e.g., D-lysine in place of L-lysine) may be used to generate polypeptides and peptidomimetics that are more stable and more resistant to endogenous proteases.

Polypeptides and peptidomimetics of the invention can be modified for in vivo use by the addition, at the amino-terminus and/or the carboxyl-terminus, of a blocking agent to decrease degradation in vivo. This can be useful in those situations in which the polypeptide termini tend to be degraded by proteases in vivo. Such blocking agents can include, without limitation, additional related or unrelated peptide sequences that can be attached to the amino and/or carboxyl terminal residues of the polypeptide or peptidomimetic of the invention. This can be done during chemical synthesis, or by recombinant DNA technology by methods familiar to artisans of average skill. Alternatively, blocking agents such as pyroglutamic acid, or other molecules known in the art, can be attached to the amino and/or carboxyl terminal residues, or the amino group at the amino terminus or carboxyl group at the carboxyl terminus can be replaced with a different moiety. Accordingly, the invention provides polypeptides and peptidomimetics that are amino-terminally and carboxyl-terminally blocked.

Polypeptides of the invention can be produced on a small or large scale through use of numerous expression systems that include, but are not limited to, cells or microorganisms that are transformed with a recombinant vector into which a polynucleotide of the invention has been inserted. Such recombinant vectors and methods for their use are described below. These vectors can be used to transform a variety of organisms. Examples of such organisms include bacteria (for example, *E. coli* or *B. subtilis*); yeast (for example, *Saccharomyces* and *Pichia*); insects (for example, baculovirus); plants; or mammalian cells (for example, COS, CHO, BHK, 293, VERO, HeLa, MDCK, W138, and NIH 3T3 cells). Also useful as host cells are primary or secondary cells obtained directly from a mammal that are transfected with a vector.

Synthetic methods may also be used to produce polypeptides and peptidomimetics of the invention. Such methods are known and have been reported (Merrifield, Science, 85:2149 (1963); Coolidge et al., U.S. Patent No. 5,595,887; Gelfand et al., U.S. Patent No. 5,116,750; Meade et al., U.S. Patent No. 5,168,049 and Klein et al., U.S. Patent No. 5,053,133; Olson et al., Peptides, 9, 301, 307 (1988)). The solid phase peptide synthetic method is an established and widely used method, which is described in the following references: Stewart et al., Solid Phase Peptide

Synthesis, W. H. Freeman Co., San Francisco (1969); Merrifield, J. Am. Chem. Soc., 85 2149 (1963); Meienhofer in "Hormonal Proteins and Peptides," ed.; C.H. Li, Vol. 2 (Academic Press, 1973), pp. 48-267; Bavaay and Merrifield, "The Peptides," eds. E. Gross and F. Meienhofer, Vol. 2 (Academic Press, 1980) pp. 3-285; and Clark-Lewis et al., Meth. Enzymol., 287, 233 (1997). Polypeptides can be readily purified by fractionation on immunoaffinity or ion-exchange columns; ethanol precipitation; reverse phase HPLC; chromatography on silica or on an anion-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; ligand affinity chromatography, and the like. Polypeptides can also be readily purified through binding of a fusion polypeptide to separation media, followed by cleavage of the fusion polypeptide to release a purified polypeptide. For example, a fusion polypeptide that includes a factor Xa cleavage site between the polypeptide and the carrier polypeptide can be created. The fusion polypeptide can be bound to an affinity column to which the carrier polypeptide portion of the fusion polypeptide binds. The fusion polypeptide can then be cleaved with factor Xa to release the polypeptide. Such a system has been used in conjunction with a factor Xa removal kit for purification of the polypeptides of the invention.

Polynucleotides, nucleic acid constructs, and expression cassettes

The invention provides polynucleotides that encode the polypeptides of the invention. The term "polynucleotide" refers broadly to a polymer of two or more nucleotides covalently linked in a 5' to 3' orientation. The terms nucleic acid, nucleic acid molecule, and oligonucleotide and protein included within the definition of polynucleotide and these terms are used interchangeably. It should be understood that these terms do not connote a specific length of a polymer of nucleotides, nor are they intended to imply or distinguish whether the polynucleotide is produced using recombinant techniques, chemical or enzymatic synthesis, or is naturally occurring.

Polynucleotides can be single-stranded or double-stranded, and the sequence of the second, complementary strand is dictated by the sequence of the first strand. The term "polynucleotide" is therefore to be broadly interpreted as encompassing a single stranded nucleic acid polymer, its complement, and the duplex formed thereby. "Complementarity" of polynucleotides refers to the ability of two single-stranded

polynucleotides to base pair with each other, in which an adenine on one polynucleotide will base pair with a thymidine (or uracil, in the case of RNA) on the other, and a cytidine on one polynucleotide will base pair with a guanine on the other. Two polynucleotides are complementary to each other when a nucleotide sequence in one polynucleotide can base pair with a nucleotide sequence in a second polynucleotide. For instance, 5'-ATGC and 5'-GCAT are fully complementary, as are 5'-GCTA and 5'-TAGC.

Preferred polynucleotides of the invention include polynucleotides having a nucleotide sequence that is "substantially complementary" to (a) a nucleotide sequence that encodes a polypeptide according to the invention, or (b) the complement of such nucleotide sequence. "Substantially complementary" polynucleotides can include at least one base pair mismatch, such that at least one nucleotide present on a second polynucleotide, however the two polynucleotides will still have the capacity to hybridize. For instance, the middle nucleotide of each of the two DNA molecules 5'-AGCAAATAT and 5'-ATATATGCT will not base pair, but these two polynucleotides are nonetheless substantially complementary as defined herein. Two polynucleotides are substantially complementary if they hybridize under hybridization conditions exemplified by 2X SSC (SSC: 150mM NaCl, 15 mM trisodium citrate, pH 7.6) at 55°C. Substantially complementary polynucleotides for purposes of the present invention preferably share at least one region of at least 20 nucleotides in length which shared region has at least 60% nucleotide identity, preferably at least 80% nucleotide identity, more preferably at least 90% nucleotide identity and most preferably at least 95% nucleotide identity. Particularly preferred substantially complementary polynucleotides share a plurality of such regions. Preferably the polynucleotides have a nucleotide sequence that is at least 70% identical to SEQ ID NO:21. More preferably the polynucleotides have a nucleotide sequence that is at least single unit percentages greater than 70% identical to SEQ ID NO:21, for example 71%, 72%, 73% identity, and so on to 100% identity to SEQ ID NO:21. Even more preferably, the polynucleotides have a nucleotide sequence that is at least 80% identical to SEQ ID NO:21. Still even more preferably, the polynucleotides have a nucleotide sequence that is at least 90% identical to SEQ ID NO:21. Yet still even more preferably, the polynucleotides have a nucleotide sequence that is at least 95%

identity to SEQ ID NO:21. Most preferably, the polynucleotides have a nucleotide sequence that is 100% identical to SEQ ID NO:21.

Percent identity between two polynucleotide sequences is generally determined by aligning the bases of the two polynucleotide sequences to optimize the number of identical bases along the lengths of their sequences; gaps in either or both sequences are permitted in making the alignment in order to optimize the number of identical bases, although the bases in each sequence must nonetheless remain in their proper order. The two polynucleotide sequences are preferably compared using the Blastn program, version 2.0.11, of the BLAST 2 search algorithm, also as described by Tatusova et al. (FEMS Microbiol. Lett, 174, 247-250 (1999)), and available on the world wide web at www.ncbi.nlm.nih.gov/blast.html. Preferably, the default values for all BLAST 2 search parameters are used, including reward for match = 1, penalty for mismatch = -2, open gap penalty = 5, extension gap penalty = 2, gap x_dropoff = 50, expect = 10, wordsize = 11, and optionally, filter on. Locations and levels of nucleotide sequence identity between two polynucleotide sequences can also be readily determined using CLUSTALW multiple sequence alignment software (J. Thompson et al., Nucl. Acids Res., 22:4673-4680 (1994)), available at from the world wide web at www.ebi.ac.uk/clustalw/.

It should be understood that a polynucleotide that encodes a polypeptide of the invention is not limited to a polynucleotide that contains all or a portion of naturally occurring genomic or cDNA nucleotide sequence, but also includes the class of polynucleotides that encode such polypeptides as a result of the degeneracy of the genetic code. For example, the naturally occurring polynucleotide sequence SEQ ID NO:21 is but one member of the class of nucleotide sequences that encodes a polypeptide having amino acid SEQ ID NO:20. The class of nucleotide sequences that encode a selected polypeptide sequence is large but finite, and the nucleotide sequence of each member of the class can be readily determined by one skilled in the art by reference to the standard genetic code, wherein different nucleotide triplets (codons) are known to encode the same amino acid. Likewise, a polynucleotide of the invention that encodes, for instance, a biologically active analog or isoform of an α-estrogen receptor or β-estrogen receptor, an α-estrogen receptor isoform or β-estrogen

receptor isoform, includes the multiple members of the class of polynucleotides that encode the selected polypeptide sequence.

A polynucleotide that "encodes" a polypeptide of the invention optionally includes both coding and noncoding regions, and it should therefore be understood that, unless expressly stated to the contrary, a polynucleotide that "encodes" a polypeptide is not structurally limited to nucleotide sequences that encode a polypeptide but can include other nucleotide sequences outside (i.e., 5' or 3' to) the coding region.

The polynucleotides of the invention can be DNA, RNA, or a combination thereof, and can include any combination of naturally occurring, chemically modified or enzymatically modified nucleotides. As noted above, the polynucleotide can be equivalent to a polynucleotide encoding a polypeptide of the invention and can include one or more additional nucleotides. Polynucleotides can also be optimized for expression in a certain type of cell. Examples of such cells include bacteria, yeast, mammalian cells and the like.

A polynucleotide of the invention may be inserted into a vector. A vector may include, but is not limited to, any plasmid, phagemid, F-factor, virus, cosmid, or phage. The vector may be in a double-stranded or single-stranded linear or circular form. The vector can also transform a prokaryotic or eukaryotic host either by integration into the cellular genome or exist extrachromosomally (e.g. autonomous replicating plasmid with an origin of replication). The polynucleotide in the vector can be under the control of, and operably linked to, an appropriate promoter or other regulatory sequence for transcription in vitro or in a host cell, such as a eukaryotic cell, or a microbe, e.g. bacteria. A regulatory sequence refers to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Examples of regulatory sequences include enhancers, promoters, translation leader sequences, introns, and polyadenylation signal sequences. They include natural and synthetic sequences as well as sequences that may be a combination of synthetic and natural sequences. Regulatory sequences are not limited to promoters. However, some suitable regulatory sequences useful in the present invention will include, but are not

limited to, constitutive promoters, tissue-specific promoters, development- specific promoters, inducible promoters and viral promoters.

The vector may be a shuttle vector that functions in multiple hosts. The vector may also be a cloning vector which typically contain one or a small number of restriction endonuclease recognition sites at which foreign DNA sequences can be inserted in a determinable fashion. Such insertion can occur without loss of essential biological function of the cloning vector. A cloning vector may also contain a marker gene that is suitable for use in the identification and selection of cells transformed with the cloning vector. Examples of marker genes are tetracycline resistance or ampicillin resistance. Many cloning vectors are commercially available (for instance Stratagene, New England Biolabs, Clonetech). A vector may be an expression vector that contains regulatory sequences which direct the expression of a polynucleotide that is inserted into the expression vector. Numerous vectors are commercially available and are known in the art (Stratagene, La Jolla, CA; New England Biolabs, Beverly, MA).

Methods to introduce a polynucleotide into a vector are well known in the art (Sambrook et al., Molecular Cloning: A Laboratory Manual, 3rd edition, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (2001)). Briefly, a vector into which a polynucleotide is to be inserted is treated with one or more restriction enzymes (restriction endonuclease) to produce a linearized vector having a blunt end, a "sticky" end with a 5' or a 3' overhang, or any combination of the above. The vector may also be treated with a restriction enzyme and subsequently treated with another modifying enzyme, such as a polymerase, an exonuclease, a phosphatase or a kinase, to create a linearized vector that has characteristics useful for ligation of a polynucleotide into the vector. The polynucleotide that is to be inserted into the vector is treated with one or more restriction enzymes to create a linearized segment having a blunt end, a "sticky" end with a 5' or a 3' overhang, or any combination of the above. The polynucleotide may also be treated with a restriction enzyme and subsequently treated with another DNA modifying enzyme. Such DNA modifying enzymes include, but are not limited to, polymerase, exonuclease, phosphatase or a kinase, to create a polynucleotide that has characteristics useful for ligation of a polynucleotide into the vector.

The treated vector and polynucleotide are then ligated together to form a construct containing a polynucleotide according to methods known in the art (Sambrook et al., Molecular Cloning: A Laboratory Manual, 3rd edition, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (2001)). Briefly, the treated nucleic acid fragment and the treated vector are combined in the presence of a suitable buffer and ligase. The mixture is then incubated under appropriate conditions to allow the ligase to ligate the nucleic acid fragment into the vector.

The invention also provides an expression cassette which contains a regulatory sequence capable of directing expression of a particular polynucleotide of the invention, such as SEQ ID NO:21, either in vitro or in a host cell. The expression cassette is an isolatable unit such that the expression cassette may be in linear form and functional in in vitro transcription and translation assays. The materials and procedures to conduct these assays are commercially available from, for instance, Promega Corp. (Madison, Wisconsin). For example, an in vitro transcript may be produced by placing a polynucleotide under the control of a T7 promoter and then using T7 RNA polymerase to produce an in vitro transcript. This transcript may then be translated in vitro through use of a rabbit reticulocyte lysate. Alternatively, the expression cassette can be incorporated into a vector allowing for replication and amplification of the expression cassette within a host cell or also in vitro transcription and translation of a polynucleotide.

Such an expression cassette may contain one or a plurality of restriction sites allowing for placement of the polynucleotide under the regulation of a regulatory sequence. The expression cassette can also contain a termination signal operably linked to the polynucleotide as well as regulatory sequences required for proper translation of the polynucleotide. The expression cassette containing the polynucleotide may be chimeric, meaning that at least one of its components is heterologous with respect to at least one of its other components. The expression cassette may also be one which is naturally occurring but has been obtained in a recombinant form useful for heterologous expression. Expression of the polynucleotide in the expression cassette may be under the control of a constitutive promoter or an inducible promoter that initiates transcription only when the host cell is exposed to some particular external stimulus.

The expression cassette may include in the 5'-3' direction of transcription, a transcriptional and translational initiation region, a polynucleotide and a transcriptional and translational termination region functional in vivo and /or in vitro. The termination region may be native with the transcriptional initiation region, may be native with the polynucleotide, or may be derived from another source.

A promoter is a nucleotide sequence that controls the expression of the coding sequence by providing the recognition for RNA polymerase and other factors required for proper transcription. A promoter includes a minimal promoter, consisting only of all basal elements needed for transcription initiation, such as a TATA-box and/or initiator that is a short DNA sequence comprised of a TATA- box and other sequences that serve to specify the site of transcription initiation, to which regulatory elements are added for control of expression. A promoter may be derived entirely from a native gene, or be composed of different elements derived from different promoters found in nature, or even be comprised of synthetic DNA segments. A promoter may contain DNA sequences that are involved in the binding of protein factors that control the effectiveness of transcription initiation in response to physiological or developmental conditions.

The invention also provides a vector into which an expression cassette has been inserted. The vector may be selected from, but not limited to, any vector previously described. Into this vector may be inserted an expression cassette through methods known in the art and previously described (Sambrook et al., Molecular Cloning: A Laboratory Manual, 3rd edition, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (2001)). In one embodiment, the regulatory sequences of the expression cassette may be derived from a source other than the vector into which the expression cassette is inserted. In another embodiment, a construct containing a vector and an expression cassette is formed upon insertion of a polynucleotide of the invention into a vector that itself contains regulatory sequences. Thus, an expression cassette is formed upon insertion of the polynucleotide into the vector. Vectors containing regulatory sequences are available commercially and methods for their use are known in the art (Clonetech, Promega, Stratagene).

In the case of a polypeptide or polynucleotide that is naturally occurring, it is preferred that such polypeptide or polynucleotide be isolated and, optionally, purified.

An "isolated" polypeptide or polynucleotide is one that is separate and discrete from its natural environment. A "purified" polypeptide or polynucleotide is one that is at least 60% free, preferably 75% free, and most preferably 90% free from other components with which they are naturally associated. Polypeptides and nucleotides that are produced outside the organism in which they naturally occur, e.g., through chemical or recombinant means, are considered to be isolated and purified by definition, since they were never present in a natural environment.

The invention further provides methods for making a polypeptide of the invention; as well as methods for making the polynucleotides that encode them. The methods include biological, enzymatic, and chemical methods, as well as combinations thereof, and are well-known in the art. For example, a polynucleotide can be expressed in a host cell from using standard recombinant DNA technologies; it can be enzymatically synthesized *in vitro* using a cell-free RNA based system; or it can be synthesized using chemical technologies such as solid phase peptide synthesis, as is well-known in the art. When recombinant DNA technologies are used, the host cell can be, for example, a bacterial cell, an insect cell, a yeast cell, or a mammalian cell.

Antibodies and peptide aptamers

The invention provides antibodies and peptide aptamers that bind to the polypeptides and peptidomimetics of the invention. Antibodies, both monoclonal and polyclonal, and peptide aptamers of the invention are thought to be particularly useful for determining levels of ER-α36 expression. This information can be used to predict if a cell or patient is refractory to treatment with a therapeutic agent, such as tamoxifen, or is predisposed to develop osteoporosis.

Accordingly, the polypeptides and peptidomimetics of the invention as described herein and any portion thereof can be used as antigens to produce antibodies, including vertebrate antibodies, hybrid antibodies, chimeric antibodies, humanized antibodies, altered antibodies, univalent antibodies, monoclonal and polyclonal antibodies, Fab proteins and single domain antibodies, as well as peptide aptamers. For example, a polypeptide having SEQ ID NO:1 or a portion thereof can be used to generate antibodies that specifically bind to ER-α36. If the polypeptides

are not sufficiently immunogenic, they can be modified by covalently linking them to an immunogenic carrier, such as keyhole limpet hemocyanin (KLH), bovine serum albumin, ovalbumin, mouse serum albumin, rabbit serum albumin, and the like.

If polyclonal antibodies are desired, a selected animal (*e.g.*, mouse, rabbit, goat, horse or bird, such as chicken) is immunized with the desired antigen. Serum from the immunized animal is collected and treated according to known procedures. If serum containing polyclonal antibodies to a polypeptide of the invention contains antibodies to other antigens, the polyclonal antibodies can be purified by immunoaffinity chromatography. Techniques for producing and processing polyclonal antisera are known in the art (see for example, Mayer and Walker eds. Immunochemical Methods in Cell and Molecular Biology (Academic Press, London) (1987), Coligan, et al., Unit 9, Current Protocols in Immunology, Wiley Interscience (1991), Green et al., Production of Polyclonal Antisera, in Immunochemical Protocols (Manson, ed.), pages 1-5 (Humana Press 1992); Coligan et al., Production of Polyclonal Antisera in Rabbits, Rats, Mice and Hamsters, in Current Protocols in Immunology, section 2.4.1 (1992)).

Monoclonal antibodies directed against the polypeptides or peptidomimetics of the invention can also be readily produced by one skilled in the art. The general methodology for making monoclonal antibodies by hybridomas is well known. Immortal antibody-producing cell lines can be created by cell fusion, and also by other techniques such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus (See Monoclonal Antibody Production. Committee on Methods of Producing Monoclonal Antibodies, Institute for Laboratory Animal Research, National Research Council; The National Academies Press; (1999), Kohler & Milstein, Nature, 256:495 (1975); Coligan et al., sections 2.5.1-2.6.7; and Harlow et al., Antibodies: A Laboratory Manual, page 726 (Cold Spring Harbor Pub. 1988)). Panels of monoclonal antibodies produced against the polypeptides and peptidomimetics of the invention can be screened for various properties, for example epitope affinity.

As an example of one procedure for creating monoclonal antibodies, antigen is emulsified in Complete Freund's Adjuvant and the emulsion used to immunize Balb/c mice (about 50-100 μg antigen per mouse given intraperitoneally). Mice are boosted

with an emulsion of antigen-Incomplete Freund's Adjuvant twice at about 10 day intervals (about 50-100 μg antigen each, given intraperitoneally). About ten days after the second booster, an antigen-capture ELISA may be run to determine the response of the mice to the antigen. The ELISA is performed by using the antigen to coat wells of microtiter plates. After overnight incubation, coated plates are washed thoroughly, and nonspecific binding sites are blocked. After incubation, plates are thoroughly washed. The primary antibody, i.e. antibody contained in the sera from the immunized mice, is diluted and added to the microtiter plate wells. Following additional washes, a goat anti-mouse IgG- and IgM- alkaline phosphatase conjugate is added to the wells. After incubation and thorough washing, the substrate for the phosphatase, p-nitrophenyl phosphate, is added to the wells. Plates are incubated in the dark for about 10-45 minutes. Subsequently, changes in absorbance of the plate's contents are read at 405 nm with a microplate spectrophotometer as an indication of mouse response to antigen. With the identification of a positive antibody, production of monoclonal antibodies can proceed. If a positive antibody is not identified, more boosters may be used, or techniques to increase the immunogenicity of the polypeptide can be implemented as stated above.

Responding mice are given a final booster consisting of about 5-100 μg, preferably 25-50 μg of antigen, preferably without adjuvant, administered intravenously. Three to five days after final boosting, spleens and sera are harvested from all responding mice, and sera is retained for use in later screening procedures. Spleen cells are harvested by perfusion of the spleen with a syringe. Spleen cells are collected, washed, counted and the viability determined via a viability assay. Spleen and SP2/0 myeloma cells (ATCC, Rockville, MD) are screened for HAT sensitivity and absence of bacterial contamination. The screening involves exposing the cells to a hypoxanthine, aminopterin, and thymidine selection (HAT) medium in which hybridomas survive but not lymphocytes or myeloma cells). The cells are combined, the suspension pelleted by centrifugation, and the cells fused using polyethylene glycol solution. The "fused" cells are resuspended in HT medium (RPMI supplemented with 20 % fetal bovine serum (FBS), 100 units of penicillin per ml, 0.1 mg of streptomycin per ml, 100 μM hypoxanthine, 16 μM thymidine, 50 μM 2-

mercaptoethanol and 30 % myeloma-conditioned medium) and distributed into the wells of microtiter plates. Following overnight incubation at 37°C in 5% CO_2, HAT selection medium (HT plus 0.4 μM aminopterin) is added to each well and the cells fed according to accepted procedures known in the art. In approximately 10 days, medium from wells containing visible cell growth are screened for specific antibody production by ELISA. Only wells containing hybridomas making antibody with specificity to the antigen are retained. The ELISA is performed as described above, except that the primary antibody added is contained in the hybridoma supernatants. Appropriate controls are included in each step.

This process generates several hybridomas producing monoclonal antibodies to the polypeptide or peptidomimetic antigen. Hybridoma cells from wells testing positive for the desired antibodies are cloned by limiting dilution and re-screened for antibody production using ELISA. Cells from positive wells are subcloned to ensure their monoclonal nature. The most reactive lines are then expanded in cell culture and samples are frozen in 90% FBS-10% dimethylsulfoxide. Monoclonal antibodies can be characterized using a commercial isotyping kit (BioRad Isotyping Panel, Oakland, CA) and partially purified with ammonium sulfate precipitation followed by dialysis. Further purification can be performed using protein-A affinity chromatography.

Antibodies can also be prepared through use of phage display techniques. In one example, an organism is immunized with an antigen, such as a polypeptide or peptidomimetic of the invention. Lymphocytes are isolated from the spleen of the immunized organism. Total RNA is isolated from the splenocytes and mRNA contained within the total RNA is reverse transcribed into complementary deoxyribonucleic acid (cDNA). The cDNA encoding the variable regions of the light and heavy chains of the immunoglobulin is amplified by polymerase chain reaction (PCR). To generate a single chain fragment variable (scFV) antibody, the light and heavy chain amplification products may be linked by splice overlap extension PCR to generate a complete sequence and ligated into a suitable vector. *E. coli* are then transformed with the vector encoding the scFV, and are infected with helper phage, to produce phage particles that display the antibody on their surface. Alternatively, to generate a complete antigen binding fragment (Fab), the heavy chain amplification product can be fused with a nucleic acid sequence encoding a phage coat protein, and

the light chain amplification product can be cloned into a suitable vector. *E. coli* expressing the heavy chain fused to a phage coat protein are transformed with the vector encoding the light chain amplification product. The disulphide linkage between the light and heavy chains are established in the periplasm of *E. coli*. The result of this procedure is to produce an antibody library with up to 10^9 clones. The size of the library can be increased to 10^{18} phages by later addition of the immune responses of additional immunized organisms that may be from the same or different hosts.

Antibodies that recognize a specific antigen can be selected through panning. Briefly, an entire antibody library can be exposed to an immobilized antigen against which antibodies are desired. Phage that do not express an antibody that binds to the antigen are washed away. Phage that express the desired antibodies are immobilized on the antigen. These phage are then eluted and again amplified in *E. coli*. This process can be repeated to enrich the population of phage that express antibodies that specifically bind to the antigen. After phage are isolated that express an antibody that binds to an antigen, a vector containing the coding sequences for the antibody can be isolated from the phage particles and the coding sequences can be recloned into a suitable vector to produce an antibody in soluble form. Phage display methods to isolate antigens and antibodies are known in the art and have been described (Gram et al., Proc. Natl. Acad. Sci., 89:3576 (1992); Kay et al., Phage display of peptides and proteins: A laboratory manual. San Diego: Academic Press (1996); Kermani et al., Hybrid, 14:323 (1995); Schmitz et al., Placenta, 21 Suppl. A:S106 (2000); Sanna et al., Proc. Natl. Acad. Sci., 92:6439 (1995)). An antibody of the invention may be derived from a "humanized" monoclonal antibody. Humanized monoclonal antibodies are produced by transferring mouse complementarity determining regions from heavy and light variable chains of the mouse immunoglobulin into a human variable domain, and then substituting human residues in the framework regions of the murine counterparts. The use of antibody components derived from humanized monoclonal antibodies obviates potential problems associated with the immunogenicity of murine constant regions. General techniques for cloning murine immunoglobulin variable domains are described (Orlandi et al., Proc. Nat'l Acad. Sci. USA, 86:3833 (1989) which is hereby incorporated in its entirety by reference).

Techniques for producing humanized monoclonal antibodies are described (Jones et al., Nature, 321:522 (1986); Riechmann et al., Nature, 332:323 (1988); Verhoeyen et al, Science, 239:1534 (1988); Carter et al., Proc. Nat'l Acad. Sci. USA, 89:4285 (1992); Sandhu, Crit. Rev. Biotech., 12:437 (1992); and Singer et al., J. Immunol., 150:2844 (1993)).

In addition, antibodies of the present invention may be derived from a human monoclonal antibody. Such antibodies are obtained from transgenic mice that have been "engineered" to produce specific human antibodies in response to antigenic challenge. In this technique, elements of the human heavy and light chain loci are introduced into strains of mice derived from embryonic stem cell lines that contain targeted disruptions of the endogenous heavy and light chain loci. The transgenic mice can synthesize human antibodies specific for human antigens, and the mice can be used to produce human antibody-secreting hybridomas. Methods for obtaining human antibodies from transgenic mice are described (Green et al., Nature Genet., 7:13 (1994); Lonberg et al., Nature, 368:856 (1994); and Taylor et al., Int. Immunol., 6:579 (1994)).

Antibody fragments of the invention can be prepared by proteolytic hydrolysis of the antibody or by expression in *E. coli* of DNA encoding the fragment. Antibody fragments can be obtained by pepsin or papain digestion of whole antibodies by conventional methods. For example, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted F(ab')2. This fragment can be further cleaved using a thiol reducing agent, and optionally a blocking group for the sulfhydryl groups resulting from cleavage of disulfide linkages, to produce 3.5S Fab' monovalent fragments. Alternatively, an enzymatic cleavage using pepsin produces two monovalent Fab' fragments and an Fc fragment directly. These methods are described (Haber, U.S. patent No. 4,036,945; Goldenberg, U.S. patent No. 4,331,647; and Thorpe et al., U.S. patent No. 6,342,221, and references contained therein; Porter, Biochem. J., 73:119 (1959); Edelman et al., Methods in Enzymology, Vol. 1, page 422 (Academic Press 1967); and Coligan et al. at sections 2.8.1-2.8.10 and 2.10.1-2.10.4).

Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light-heavy chain fragments, further cleavage of fragments, or other

enzymatic, chemical, or genetic techniques may also be used, so long as the fragments bind to the antigen that is recognized by the intact antibody.

For example, Fv fragments comprise, an association of V_H and V_L chains. This association may be noncovalent (Inbar et al., Proc. Nat'l Acad. Sci. USA, 69:2659 (1972)). Alternatively, the variable chains can be linked by an intermolecular disulfide bond or cross-linked by chemicals such as glutaraldehyde (Sandhu, Crit. Rev. Biotech., 12:437 (1992)). Preferably, the Fv fragments comprise V_H and V_L chains connected by a peptide linker. These single-chain antigen binding proteins (sFv) are prepared by constructing a structural gene comprising DNA sequences encoding the V_H and V_L domains connected by an oligonucleotide. The structural gene is inserted into an expression vector, which is subsequently introduced into a host cell such as *E. coli*. The recombinant host cells synthesize a single polypeptide chain with a linker peptide bridging the two V domains. Methods for producing sFvs are described (Whitlow et al., Methods: A Companion to Methods in Enzymology, Vol. 2, page 97 (1991); Bird et al., Science, 242:423 (1988), Ladner et al., U.S. patent No. 4,946,778; Pack et al., Bio/Technology, 11:1271 (1993); and Sandhu, Crit. Rev. Biotech., 12:437 (1992)).

Another form of an antibody fragment is a peptide that forms a single complementarity-determining region (CDR). CDR peptides ("minimal recognition units") can be obtained by constructing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction to synthesize the variable region from RNA of antibody-producing cells (Larrick et al., Methods: A Companion to Methods in Enzymology, Vol. 2, page 106 (1991)).

The invention also provides peptide aptamers that bind to the polypeptides or peptidomimetics of the invention. Peptide aptamers are polypeptides that bind to a polypeptide or peptidomimetic of the invention with affinities that are often comparable to those for monoclonal antibody-antigen complexes. In one example, peptide aptamers can be isolated according to mRNA display through use of a DNA library that contains a promoter, a start codon, a nucleic acid sequence coding for random peptides, and a nucleic acid sequence that codes for a histidine tag. This library is transcribed using a suitable polymerase, such as T7 RNA polymerase, after

which a puromycin-containing poly A sequence is ligated onto the 3' end of the newly formed mRNAs. When these mRNAs are translated *in vitro*, the nascent peptides form covalent bonds to the puromycin of the poly A sequence to form an mRNA-peptide fusion molecule. The mRNA-peptide fusion molecules are then purified through use of Ni-NTA agarose and oligo-dT-cellulose. The mRNA portion of the fusion molecule is then reverse transcribed. The double-stranded DNA/RNA-peptide fusion molecules are then incubated with a peptide of the invention and unbound fusion molecules are washed away. The bound fusion molecules are eluted from the immobilized peptides and are then amplified by PCR. This process may be repeated to select for peptide aptamers having high affinity for the polypeptides of the invention. The sequence of the nucleic acid coding for the peptide aptamers can then be determined and cloned into a suitable vector. Methods for the preparation of peptide aptamers have been described (Wilson et al., Proc. Natl. Acad. Sci., 98:3750 (2001)). Accordingly, the invention provides peptide aptamers that recognize polypeptides and peptidomimetics of the invention.

Antibodies and peptide aptamers can be screened to determine the identity of the epitope to which they bind. An epitope refers to the site on an antigen, such as a polypeptide of the invention, to which the paratope of an antibody binds. An epitope usually consists of chemically active surface groupings of molecules, such as amino acids or sugar side chains, and can have specific three-dimensional structural characteristics, as well as specific charge characteristics. Methods which can be used to identify an epitope are known in the art (Harlow et al., Antibodies: A Laboratory Manual, page 319 (Cold Spring Harbor Pub. 1988).

Antibodies and peptide aptamers may be screened for their ability to specifically bind to a polypeptide or peptidomimetic of the invention. For example, antibodies or peptide aptamers that specifically bind to the ER-α36 isoform, but not the ER-α46 or α66 isoform, can be selected through use of methods routine in the art. Briefly, a buffer containing the antibodies or peptide aptamers can be applied to a column containing immobilized ER-α36. The column can be washed to remove antibodies or peptide aptamers that do not bind to the ER-α36 isoform. The antibodies or peptide aptamers can then be eluted from the column through use of

buffer having a high salt concentration. The buffer containing the eluted antibodies or peptide aptamers is then dialyzed to lower the salt concentration. The dialyzed buffer containing the antibodies or peptide aptamers is then applied to a column containing immobilized estrogen receptor ER-α46 and ER-α66 isoform. Antibodies or peptide aptamers that bind to the ER-α46 or ER-α66 isoform are retained on the column while antibodies or peptide aptamers which bound the ER-α36 isoform, but which do not bind the ER-α46 or ER-α66 isoform, will flow through the column. These antibodies or peptide aptamers can be collected and used to specifically detect the ER-α36 isoform. This procedure can be used with any combination of polypeptides or portions thereof to select for antibodies and peptide aptamers. Numerous other methods may be used to select antibodies and peptide aptamers that specifically bind to an individual polypeptide. Such methods are known and are routine to those of skill in the art (see Kitajima et al., U.S. Patent No. 6,534,281).

Accordingly, the invention provides antibodies and peptide aptamers that are able to cross-react with the polypeptides of the invention. In addition, the invention provides antibodies and peptide aptamers that are able to specifically bind to the ER-α36 isoform, without cross-reacting with other polypeptides.

The antibodies and peptide aptamers of the invention may be coupled to a large variety of detectable markers. Examples of such detectable markers include fluorescent markers, enzymes, radioisotopes, and the like. Methods to couple antibodies and peptide aptamers to detectable markers are known in the art and have been described (see Kitajima et al., U.S. Patent No. 6,534,281). Such labeled antibodies and peptide aptamers are useful within automated systems for detection of tamoxifen refractory cells. The antibodies and peptide aptamers can also be coupled to toxins.

Compositions

The invention provides compositions that can be used for the administration of polypeptides, peptidomimetics, antibodies, and peptide aptamers of the invention to a patient in need thereof. In one example, a composition can contain a polypeptide or peptidomimetic of the invention, and optionally a pharmaceutically acceptable carrier.

In another example, a composition can contain an antibody or peptide aptamer of the invention, and optionally a pharmaceutically acceptable carrier.

The compositions of the invention may be prepared in many forms that include tablets, hard or soft gelatin capsules, aqueous solutions, suspensions, and liposomes and other slow-release formulations, such as shaped polymeric gels. An oral dosage form may be formulated such that the polypeptide, peptidomimetic, antibody, or peptide aptamer is released into the intestine after passing through the stomach. Such formulations are described in Hong et al., U.S. Patent No. 6,306,434 and in the references contained therein.

Oral liquid compositions may be in the form of, for example, aqueous or oily suspensions, solutions, emulsions, syrups or elixirs, or may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid compositions may contain conventional additives such as suspending agents, emulsifying agents, non-aqueous vehicles (which may include edible oils), or preservatives.

A polypeptide, peptidomimetic, antibody, or peptide aptamer can be formulated for parenteral administration (e.g., by injection, for example, bolus injection or continuous infusion) and may be presented in unit dosage form in ampules, prefilled syringes, small volume infusion containers or multi-dose containers with an added preservative. The compositions may take such forms as suspensions, solutions, or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Compositions suitable for rectal administration can be prepared as unit dose suppositories. Suitable carriers that may be included in the composition include those exemplified by saline solutions and other materials commonly used in the art.

For administration by inhalation, a polypeptide, peptidomimetic, antibody or peptide aptamer can be conveniently delivered from an insufflator, nebulizer or a pressurized pack or other convenient means of delivering an aerosol spray. Pressurized packs may comprise a suitable propellant such as dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount.

Alternatively, for administration by inhalation or insufflation, a polypeptide, peptidomimetic, antibody, or peptide aptamer may take the form of a dry powder composition, for example, a powder mix of a modulator and a suitable powder base such as lactose or starch. The powder composition may be presented in unit dosage form in, for example, capsules or cartridges or, e.g., gelatin or blister packs from which the powder may be administered with the aid of an inhalator or insufflator. For intra-nasal administration, a polypeptide, antibody, peptidomimetic or peptide aptamer may be administered via a liquid spray, such as via a plastic bottle atomizer.

A polypeptide, peptidomimetic, antibody or peptide aptamer can be formulated for transdermal administration. A polypeptide, peptidomimetic, antibody, or peptide aptamer can also be formulated as an aqueous solution, suspension or dispersion, an aqueous gel, a water-in-oil emulsion, or an oil-in-water emulsion. A transdermal formulation may also be prepared by encapsulation of a polypeptide, peptidomimetic, antibody, or peptide aptamer within a polymer, such as those described in Uhrich U.S. Patent No. 6,365,146. The dosage form may be applied directly to the skin as a lotion, cream, salve, or through use of a patch. Examples of patches that may be used for transdermal administration are described in Chien et al., U.S. Patent. No. 5,560,922 and Chien et al., U.S. Patent. No. 5,788,983.

Compositions of the invention may also contain other ingredients such as flavorings, colorings, anti-microbial agents, asnd preservatives. In addition, a composition of the invention can include pharmaceutically active ingredients, such as hormones, anti-necrotic agents, vasodilators, pharmaceutical agents and the like.

Examples of pharmaceutical agents include Ace inhibitors, adrenergic agonists, adrenergic blockers, adrenocortical steroids, adrenocortical suppressants, adrenocorticotropic hormones, alcohol deterrents, aldose reductase inhibitors, aldosterone antagonists, reductase inhibitors, AMPA receptor antagonists, anabolic steroids, analeptics, analgesics, androgens, anesthetics, antihypertensives, anorexics, antacids, anthelmintics, antiacne compounds, antiallergics, antialopecia agents, antiameobic, antiandrogens, antianginals, antiarrhythmics, antiarteriosclerotics, antiarthritics, antirheumatics, antiasthmatics, antibiotics, antineoplastics, anticholelithogenics, anticholesteremics, anticoagulants, anticonvulsants, antidepressants, antidiabetics, antidiarrheals, antidiuretics, antidotes, antidyskinetics,

antieczematics, antiemetics, antiepileptics, antiestrogens, antifibrotics, antiflatulants, antifungals, antiglaucomas, antigonadotropins, antigouts, antihemophilic factors, antihemorrhagics, antihistamines, antihypercholesterolemics, antihyperlipidemics, antihyperlipoproteinimics, antihyperparathyroids, antihyperphosphatemics, antihypertensives, antihyperthyroids, antihypotensives, antihypothyroids, anti-infectives, anti-inflammatories, antileprotic, antileukemics, antilipemics, antilipidemics, antimalarials, antimanics, antimethemoglabbinemics, antimigraines, antimuscarinics, antimycotics, antinauseants, antineoplastics, antineutropenics, antiobesity agents, antiobsessionals, antiosteoporotics, antipagetics, antiparkinsonians, antiperistaltics, antipheochromocytomas, antipneumocystics, antiprogestins, antiprostatic hypertrophies, antiprotozoals, antipruritics, antipsoriatics, antipsychotics, antipyretics, antirheumatics, antirickettsials, antiseborrheics, antisepsis agents, antiseptics, disinfectants, antispasmotics, antisyphilitics, antithrombocythemics, antithrombotics, antituberculars, antitumor agents, antitussives, antiulceratives, antiurolothics, antivenins, antivertigo agents, antivirals, anxiolytics, aromatase inhibitors, astringents, antriopeptidase inhibitors, benzodiazepine antagonists, beta-blockers, bone resorption inhibitors, bradycardiac agents, bradykinin antagonists, bronchodilators, calcium channel blockers, calcium regulators, calcium supplements, carbonic anhydrase inhibitors, cardiac depressants, cardioprotectives, cardiotonics, cathartics, CCK antagonists, chelating agents, cholelitholytic agents, choleretics, cholinergics, cholinesterase inhibitors, cholinesterase reactivators, CNS stimulants, cognitive activators, COMT inhibitors, contraceptives, cyclooxygenase-2 inhibitors, cytoprotectants, debriding agents, decongestants, dental plaque inhibitors, depigmentors, dermatitis herpetiforms suppressants, digestive aids, diuretics, dopamine receptor agonists, dopamine receptor antagonists, ectoparasiticides, emetics, endotherin receptor antagonists, enkephalinase inhibitors, enzymes, enzyme cofactors, enzyme inducers, estrogens, estrogen antagonists, expectorants, fibrinogen receptor antagonists, gastric and pancreatic secretion stimulants, gastric proton pump inhibitors, gastric secretion inhibitors, gastroprokenetics, glucocorticoids, alpha-glucosidase inhibitors, gonad-stimulating agents, growth hormone antagonists, growth hormone inhibitors, growth hormone releasing factors, growth stimulation agents, hematinics, hematopoietics, hemolytics, hemorheologic agents, hemostatic agents,

heparin antagonists, hepatoprotectants, histamine H1-receptor antagonists, histamine H2-receptor antagonists, anti-HIV agents, HMG COA reductase inhibitors, hypnotic agents, immunomodulators, immunosuppressants, insulin sensitizers, keratolytics, lactation stimulating hormones, laxatives, leukotriene antagonists, LH-RH agonists, LH-RH antagonists, lipotropics, 5-lipoxygenase inhibitors, lupus erythematosus suppressants, tranquilizers, metalloprotease inhibitors, mineralocorticoids, miotics, monoamine oxidase inhibitors, mucolytics, muscle relaxants, mydriatics, narcotics, narcotic antagonists, nasal decongestants, neuraminidase inhibitors, neuroleptics, neuromuscular blocking agents, neutral endopeptidase inhibitors, neuroprotectives, NMDA receptor antagonists, nootropics, ovarian hormones, oxytocics, pepsin inhibitors, phosphodiesterase inhibitors, pigmentation agents, plasma volume expanders, platelet activation factor agonists, potassium channel activators, potassium channel blockers, pressor agents, progesterones, prolactin inhibitors, prostaglandins, prostaglandin analogs, protease inhibitors, pulmonary surfactants, respiratory stimulants, reverse transcriptase inhibitors, sclerosing agents, sedatives, serenics, serotonin noradrenaline reuptake inhibitors, serotonin receptor agonists, serotonin receptor antagonists, serotonin reuptake inhibitors, sialagogues, somatostatin analogs, succinylcholine synergists, thrombolytics, thromboxane A2-receptor antagonists, thromboxane A2-synthetase inhibitors, thyroid hormones, thyroid inhibitors, thyrotropic hormones, tocolytics, topical protectants, topoisomerase I inhibitors, topoisomerase II inhibitors, tranquilizers, uricosurics, vasodilators, vasopeptidase inhibitors, vasoprotectants, vulnerarys, xanthine oxidase inhibitors, and the like. Numerous therapeutic agents are known in the art (Merck Index, Merck Research Laboratories, 13th edition, Whitehouse Station, NJ (2001); Physicians Desk Reference, Thompson PDR, 58th edition, Des Moines, IA (2004); Mosbys 2004 Drug Guide, Mosby Inc., St. Louis, MO (2004)).

It will be appreciated that the amount of a polypeptide, peptidomimetic, antibody, or peptide aptamer required for use in treatment will vary not only with the particular carrier selected but also with the route of administration, the nature of the condition being treated and the age and condition of the patient. Ultimately the attendant health care provider may determine proper dosage. In addition, a composition of the invention may be formulated as a single unit dosage form.

Method to determine if a cell responds, or is refractory, to treatment with a therapeutic agent

In one example, the invention provides methods to determine if a cell, or tissue containing such cells, is refractory to treatment with a therapeutic agent. Such therapeutic agents are generally thought to promote or inhibit estrogen signaling and their action is therefore either directly or indirectly dependent on the activity of an estrogen receptor.

A therapeutic agent may be exemplified by tamoxifen. For example, a cell is considered to be refractory to treatment with tamoxifen if treatment of the cell with tamoxifen does not decrease proliferation of the cell, or if such treatment causes an increase in cellular proliferation. The method involves comparing the ratio of ER-α36 to ER-α46, the ratio of ER-α36 to ER-α66, the ratio of ER-α36 to ER-β, or any combination thereof, in a test cell to the corresponding ratio in a control cell. Such a ratio is referred to herein as an ER-α36 ratio. In one example, the control cell can be a cell that is refractory to treatment with tamoxifen. In another example, the control cell is a cell that is not refractory to tamoxifen treatment. If an above described ER-α36 ratio determined in the test cell is the same as the ER-α36 ratio described in the control cell, then the test cell is classified according to the status of the control cell. For example, if the ER-α36 to ER-α66 ratio of the test cell is the same as the ER-α36 to ER-α66 ratio in a control cell known to be refractory to tamoxifen treatment, then the test cell is classified as being refractory to tamoxifen treatment. However, if the ER-α36 to ER-α66 ratio in the test cell is the same as the ER-α36 to ER-α66 ratio in a control cell that is not refractory to tamoxifen treatment, then the test cell is classified as not being refractory to tamoxifen treatment. In another example, the ER-α36 ratio determined in a test cell is compared to the corresponding ratio in a control cell known to be refractory to tamoxifen treatment, and to the ratio in a control cell that is known not to be refractory to tamoxifen treatment. The test cell is then classified as being refractory to tamoxifen treatment, or not refractory to tamoxifen treatment, as described above. An example of a control cell that is known not to be refractory to tamoxifen treatment is MCF7 (ATTC Collection accession number HTB-

22). Breast cancer cells that are known to be refractory to tamoxifen treatment can also be used as control cells by comparing the expression level of an ER-α isoform in the breast cancer cell control to the ER-α isoform level in the test cells.

A test cell may be cultured in vitro according to methods known in the art. Alternatively, a test cell may be obtained from a living organism through, for example, biopsy. For example, test cells may be obtained through biopsy of human breast or uterine tissue. Samples obtained from nearly any type of tissue may be used within the method of the invention. Control cells may be cultured in vitro according to methods known in the art. Control cells may also be obtained from tissue samples through, for example, biopsy.

The expression level of ER-α36, and the ratio of ER-α36 to ER-α46, ER-α66, or ER-β in cells, can be determined through use of numerous standard assays. Examples of immunoassays include competitive and non-competitive assays such as a radioimmunoassay, immunoenzymometric assay, immunofluorometric assay, or enzymoimmunassays assays. Chemiluminescent methods with horseradish peroxidase, alkaline phosphatase, or other chemiluminescent detection agents can also be used. Western blotting and chromatographic assays can also be used within the method of the invention. The antibodies and peptide aptamers of the invention can be used within such assays to detect the presence of ER-α36, or fragments thereof, in a cell or tissue. Antibodies that bind to ER-α46, ER-α66, and ER-β have been described.

The same method may be used to determine if a cell is refractory to treatment with numerous therapeutic agents. Examples of such agents include centchroman, delmadinone acetate, tamoxifen, droloxifene, idoxifene, raloxifene, toremifene, a bisphosphonate, calcitonin, tribolone, parathyroid hormone, strontium ranelate, a growth factor, a cytokinem, and the like.

Method to increase or decrease expression of an estrogen regulated gene in a cell

The invention provides a method to increase or decrease expression of an estrogen regulated gene in a cell that involves contacting the cell with an agent that modulates expression of the estrogen regulated gene. An estrogen regulated gene is

broadly defined as a gene having expression that is either increased or decreased within a cell in response to contact of the cell with estrogen. Examples of such agents include a polypeptide, a peptidomimetic, an antibody, a peptide aptamer, or a composition, of the invention. Additional agents can also be used such as anti-estrogens, synthetic or natural estrogens, and like molecules thereof. These agents can also be contacted with the cell in any combination.

In one example, expression of an estrogen regulated gene can be increased by contacting the cell with an antibody or peptide aptamer that specifically binds to ER-α36. It is thought that contacting ER-α36 with such an antibody or peptide aptamer will block ER-α36 from inhibiting the action of endogenous estrogen receptors, such as those containing ER-α46, ER-α66 or ER-β, within the cell and will increase expression of estrogen receptor regulated genes. A composition containing an antibody, peptide aptamer, or both an antibody and peptide aptamer that bind to ER-α36 may also be used to increase expression of an estrogen receptor regulated gene.

In another example, expression of an estrogen regulated gene can be decreased by contacting the cell with a polypeptide, peptidomimetic, analog, or any combination thereof, that mimics that activity of ER-α36 to inactivate ER-α46, ER-α66, ER-β, or any combination thereof. A composition containing a polypeptide, peptidomimetic, or any combination thereof that mimics the activity of ER-α36 may also be used to decrease expression of an estrogen receptor regulated gene.

Numerous examples of genes are known that are regulated by estrogen receptors (Frasor et al., Endocrinology, 144:4562 (2003)). Additionally, methods to determine if the expression of a gene is regulated in an estrogen dependent manner are known in the art. For example, gene chip technology can be used to determine if a gene is estrogen regulated (Frasor et al., Endocrinology, 144:4562 (2003)). Additional methods include, nuclease protection assays, northern blotting, western blotting, immunoassays, and the like.

Estradiol is an example of an estrogen that up-regulates or down-regulates the expression of estrogen regulated genes. Examples of estradiol up-regulated genes, and their GeneBank accession numbers, involved in cell cycle and apoptosis include,

but are not limited to: cyclin D1 (NM_053056), cell division cycle 6 homolog (NM_001254), cell division cycle 2 (NM_001786), minichromosome maintenance deficient 5 (NM_006739), DNA polymerase alpha (NM_002689), minichromosome maintenance deficient 2 (NM_004526), membrane-associated tyrosine- and threoninespecific cdc2-inhibitory kinase (Myt1) (NM_004203), minichromosome maintenance deficient 3 (NM_002388), cyclin A2 (NM_001237), replication factor C4 (NM_002916), proliferating cell nuclear antigen (NM_002592), DNA polymerase epsilon 2 (NM_002692), survivin (NM_001168), cell division cycle 20 homolog (NM_001255), BUB1 budding uninhibited by benzimidazoles 1 homolog (NM_004336), and serine/threonine kinase 6 (AIK) (NM_003600).

Examples of estradiol down-regulated genes, and their GeneBank accession numbers, involved in cell cycle and apoptosis include, but are not limited to: BCL2-antagonist/killer 1 (NT_007592), BCL2-interacting killer (apoptosis-inducing) (NM_001197), BTG family member 2 (NM_006763), cyclin G2 (NM_004354), cyclin-dependent kinase inhibitor 1A (p21, Cip1) (NM_000389), immediate early response 3 (NM_052815), retinoblastoma-like 2 (p130) (NM_005611), checkpoint suppressor 1 (NM_005197), BTG family member 1 (NM_001731), CASP2 and RIPK1 domain containing adaptor with death domain (NM_003805), and caspase 9 apoptosis-related cysteine protease (NM_001229).

Examples of estradiol up-regulated genes, and their GeneBank accession numbers, that encode growth factors, cytokines and hormones include, but are not limited to: insulin-like growth factor binding protein 4 (NM_001552), amphiregulin (NM_001657), chemokine ligand 12 (stromal cell-derived factor 1) (NM_000609), vascular endothelial growth factor (NM_003376), stanniocalcin 2 (NM_003714), WNT1 inducible signaling pathway protein 2 (NM_003881), and insulin-like growth factor binding protein 5 (NM_000599).

Examples of estradiol down-regulated genes, and their GeneBank accession numbers, that encode growth factors, cytokines and hormones include, but are not limited to: interleukin 4 (NM_000589), pre-B-cell colony-enhancing factor (NM_005746), transforming growth factor-beta 3 (NM_003239), platelet-derived growth factor beta polypeptide (NM_002608), bone morphogenetic protein 4

(NM_001202), stanniocalcin 1 (NM_003155), and inhibin beta B (activin AB beta polypeptide) (NM_002193).

Examples of estradiol up-regulated genes, and their GeneBank accession numbers, that encode receptors and signal transduction proteins include, but are not limited to: Ret/Ptc (NM_000323), retinoic acid receptor alpha (NM_000964), RAB31 (NM_006868), RAS guanyl releasing protein 1 (NM_005739), calcitonin receptor (NM_001742), Ret/Ptc2 adenylate cyclase 9 (NM_001116), growth arrest and DNA-damage-inducible beta (NM_015675), glycine receptor beta (NM_000824), tetraspan 5 (NM_005723), prostaglandin E receptor 3 (NM_000957), Ste20-related serine/threonine kinase (NM_014720), osteoclast stimulating factor 1 (NM_012383), Janus kinase 1 (NM_002227), protein phosphatase 2 regulatory subunit A beta (NM_002716), and chemokine binding protein 2 (NM_001296).

Examples of estradiol down-regulated genes, and their GeneBank accession numbers, that encode receptors and signal transduction proteins include, but are not limited to: B-cell linker (NM_013314), chemokine (C-X-C motif) receptor 4 (NM_003467), erythropoietin receptor (NM_000121), interleukin 1 receptor type I (NM_000877), protein tyrosine phosphatase non-receptor type 12 (NM_002835), dual specificity phosphatase 4 (NM_001394), serine/threonine kinase 3 (STE20 homolog) (NM_006281), CDC42 effector protein (Rho GTPase binding) 4 (NM_012121), growth factor receptor-bound protein 10 (NM_005311), phosphoinositide-3-kinase regulatory subunit polypeptide 3 (NM_003629), Cas-Br-M (murine) ecotropic retroviral transforming sequence b (NM_004351), mitogen-activated protein kinase kinase 6 (NM_002758), Notch homolog 3 (Drosophila) (NM_000435), annexin A3 (NM_005139), coxsackie virus and adenovirus receptor (NM_001338), dual specificity phosphatase 1 (NM_004417), serum-inducible kinase (NM_006622), RAP1 GTPase activating protein 1 (NM_002885), phosphatidylinositol (4,5) bisphosphate 5-phosphatase A (NM_014422), IQ motif containing GTPase activating protein 1 (NM_003870), retinoic acid induced 3 (NM_003979), v-erb-b2 (NM_004448), T cell receptor gamma locus (NG_001336), parvalbumin (NM_002854), interferon gamma receptor 2 (interferon gamma transducer 1) (NM_005534), RAB9A member RAS oncogene family (NM_004251), discoidin domain receptor family member 1 (NM_001954), PTEN induced putative kinase 1

(NM_032409), protein phosphatase 1 regulatory (inhibitor) subunit 3C (NM_005398), son of sevenless homolog 2 (Drosophila) (NT_025892), RAB6A, member RAS oncogene family (NM_002869), low density lipoprotein receptor (NM_000527), syndecan 4 (amphiglycan, ryudocan) (NM_002999), transforming growth factor, beta receptor II (NM_003242), inositol hexaphosphate kinase 1 (NM_153273), dual specificity phosphatase 3 (NM_004090), retinoid X receptor alpha (NM_002957), protein tyrosine phosphatase receptor type K (NM_002844), leptin receptor gene-related protein (NM_017526), reticulocalbin 2 EF-hand calcium binding domain (NM_002902), phosphoinositide-3-kinase class 2 beta polypeptide (NM_002646), v-erb-b2, homolog 3 (ERBB3) (NM_001982), ras homolog gene family member E (NM_005168), dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2 (NM_003583), serine/threonine kinase 16 (NM_003691), phosphoinositol 3-phosphate-binding protein-3 (NM_014935), and GTP cyclohydrolase I feedback regulatory protein NM_005258.

Examples of estradiol up-regulated genes, and their GeneBank accession numbers, that encode transcription factors and transcriptional coregulators include, but are not limited to: homeo box C4 (NM_153633), core-binding factor runt domain alpha subunit 2 translocated to 3 (NM_005187), early growth response 3 (NT_008300), c-fos (NM_005252), nuclear receptor interacting protein 1 (NM_003489), E74-like factor 1 (NT_009984), homeo box C6 (NM_004503), homeo box C5 (NM_018953), c-myc (NM_002467), TGFB-induced factor 2 (NM_021809), ELL-related RNA polymerase II elongation factor (NM_012081), TATA element modulatory factor 1 (NM_007114), c-myb (NM_005375), retinoblastoma binding protein 7 (NM_002893), a-myb (NT_008166), b-myb (NM_002466), breast cancer 2 early onset (NM_000059), and breast cancer 1 early onset (NM_007294).

Examples of estradiol down-regulated genes, and their GeneBank accession numbers, that encode transcription factors and transcriptional coregulators include, but are not limited to: MAD mothers against decapentaplegic homolog 6 (NM_005585), core promoter element binding protein (NM_001300), inhibitor of DNA binding 2 (NM_002166), nuclear receptor coactivator 2 (NM_006540), nuclear receptor subfamily 2 group C member 1 (NM_003297), transcriptional intermediary factor 1 (NM_003852), aryl-hydrocarbon receptor nuclear translocator 2 (NM_014862), jun B

proto-oncogene (NM_002229), MAX dimerization protein 4 (NM_006454), interferon regulatory factor 6 (NM_006147), special AT-rich sequence binding protein 1 (NM_002971), delta sleep inducing peptide immunoreactor (NM_004089), HMG-box containing protein 1 (NM_012257), LPS-induced TNF-alpha factor (NM_004862), basic helix-loop-helix domain containing class B 2 (DEC1) (NM_003670), SRY (sex determining region Y)-box 9 (NM_000346), Aml1-Evi-1 (S69002), nuclear factor related to kappa B binding protein (NM_006165), inhibitor of DNA binding 1 (NM_002165), proline rich 2 (NM_006813), MAD mothers against decapentaplegic homolog 3 (NM_005902), zinc finger protein 217 (NM_006526), nuclear receptor coactivator 3 (NM_006534), transforming growth factor beta-stimulated protein TSC-22 (NM_006022), basic transcription element binding protein 1 (KLF9) (NM_001206), pre-B-cell leukemia transcription factor 1 (NM_002585), regulatory factor X 5 (NM_000449), RING1 and YY1 binding protein (NM_012234), hepatocyte nuclear factor 3 alpha (NM_004496), Ets2 repressor factor (NM_006494), forkhead box O3A (NM_001455), thyroid transcription factor 1 (NM_003317), zinc finger protein 36 C3H type-like 2 (NM_006887), iroquois homeobox protein 5 (NM_005853), and hairy homolog (Drosophila) (NM_005524).

Method to reduce or eliminate an estrogen related disorder in a mammal

The invention provides a method to reduce or eliminate an estrogen related disorder in a mammal. The method involves administering an effective amount of a polypeptide, peptidomimetic, antibody, or peptide aptamer of the invention, or any combination thereof, to the mammal in need of such treatment, and reducing or eliminating one or more symptoms of the disorder. Compositions containing any combination of polypeptides, peptidomimetics, antibodys, or peptide aptamers of the invention can also be administered to a mammal having an estrogen receptor related disorder. These compositions can contain additional therapeutic agents, such as estrogen, anti-estrogen, and the like.

Estrogen related disorders include disorders that result from an increase or decrease in estrogen receptor activity or signaling when compared to a control mammal that does not exhibit the disorder. Examples of estrogen receptor related disorders include, but are not limited to, Alzheimer's disease, Parkinson's disease,

multiple sclerosis, depression, bipolar disorder, schizophrenia, endometriosis, osteoporosis, and ischemic stroke. Estrogen receptor activity or signaling can be determined through use of many assays.

For example, the regulatory function of ER-α36 on transcription activation that is mediated by ER-α66 and ER-α46 can be evaluated by through use of co-transfection assays. A luciferase-expressing nucleic acid reporter construct that contains a thymidine kinase promoter and two estrogen response elements (sequence from –331 to –289 of the chicken Vitellogenin A2 gene) operably linked to an open reading frame that encodes luciferase can be transfected into a cell. HeLa cells, HepG2 cells and Saos2 cells are examples of cells that can be used, however, other cells may also be used. Transcription activation of luciferase activity is then measured in cells that are contacted with estradiol and in cells that are not contacted with estradiol. If the luciferase activity in the cells contacted with estradiol is greater than luciferase activity in cell that were not contacted with estradiol, then the cell is deemed to be responsive to estrogen signaling. In addition, similar assays can be conducted with tamoxifen (a partial antagonist) or ICI 182,780 (an antagonist) to determine if the signaling results from AF-1 or AF-2 (Berry et al., EMBO J., 9:2811 (1990); Tzukerman et al., Mol. Endocrin., 8:21 (1994); Norris et al., Mol. Endocrin., 11:747 (1997); Denger et al., Mol. Endocrin., 15:2064 (2001)). Cells can also be transfected with a nucleic acid construct that provides for expression of ER-α36 to determine the effect of ER-α36 on estrogen response.

Cell proliferation assays can be used to determine if cells are responsive to estrogen signaling. In one example, DNA synthesis that is stimulated by estradiol will be assayed by adding bromodeoxyuridine (BrdU) to medium in which cells are being cultured. Estradiol will then be added to the medium in which some of the cells are being cultured while estradiol will not be added to the medium in which other cells are being cultured. After about 24 hours, the cells can be fixed and stained with fluorescein-conjugated mouse anti-BrdU monoclonal antibodies (clone BMC 9318 from Boehringer Mannheim) to determine whether estradiol stimulated DNA synthesis. Such methods can be used to determine specific pathways through which estrogen signaling occurs in various cells. For example, PD98059, a selective Mek1

inhibitor, can be added to the culture medium to determine if estrogen signaling proceeds through the MAPK pathway. This method can also be used to determine if recombinant expression of ER-α36 or ER-α46 confers estrogen-stimulated cell growth in some of the tested cell lines. If so, the downstream signaling pathways possibly involved in estradiol stimulated cell proliferation can be determined, such as the effects of estradiol on ERK1/2 activity in stable cells that express ER-α36 or ER-α46. MCF7 cells can be used as a positive control, since it has been reported that exposure of estrogen-deprived MCF7 cells to estradiol is immediately followed by transient activation of ERK1/2 activity. (Kelly et. al., 2001; Migliaccio et. al., 1996). Cells can be maintained for 2-3 days in estrogen and phenol red-free medium to make estrogen-deprived cells, or can be further maintained in serum-free medium in which 0.25% cell-culture grade BSA is added to make quiescent cells. Both estrogen deprived cells and quiescent cells can be treated with 10^{-8} M estradiol in 0.01% EtOH or 0.01% EtOH alone (vehicle) for 0, 2, 10 and 60 minutes. Serum stimulation with 10% charcoal-treated fetal calf serum in serum starved cells can be used as a positive control. Western blot analysis of ERK1/2 phosphorylation status in extracts from the estradiol or serum stimulated cells will be performed using an antibody against phospho-specific ERK1/2, and with an anti ERK1/2 antibody as an internal control. This method can be used to determine whether ER-α36 or ER-α46 mediates the activation of the MAPK pathway by estrogen signaling in MCF7 cells (Kelly et. al., Trends Endocrinol. Metab., 12:152 (2001); Migliaccio et. al., EMBO J., 15:1292 (1996)) that express all three isoforms of ER-α. In addition, PD98059, a selective Mek1 inhibitor, can be used to test whether the MAPK pathway is responsible for estrogen signaling stimulated cell proliferation in these cells.

An estrogen related disorder that is caused by an increase in estrogen receptor activity or signaling can be reduced or eliminated by administering an effective amount of a polypeptide or peptidomimetic of the invention, or a composition that contains a polypeptide, a peptidomimetic, or combination thereof, to the mammal. The polypeptide or peptidomimetic is thought to inactivate estrogen receptors that are endogenously expressed within the cells of the mammal and thereby reduce or

eliminate one or more symptoms of the disorder caused by increased estrogen receptor activity or signaling.

In another example, an estrogen related disorder that is caused by a decrease in estrogen receptor activity or signaling can be reduced or eliminated by administering an effective amount of an antibody or peptide aptamer of the invention, or a composition that contains an antibody, peptide aptamer, or combination thereof, to the mammal. The antibody or peptide aptamer is thought to increase endogenous estrogen receptor activity within the cells of the mammal by blocking the inhibitory activity of ER-α36 on the endogenous estrogen receptors, such as ER-α46, ER-α66 or ER-β. It is thought that decreasing or eliminating the inhibitory activity of ER-α36 will reduce or eliminate one or more symptom of a disorder caused by decreased estrogen receptor activity or signaling.

Method to reduce or eliminate estrogen receptor promoted proliferation or replication of a cell

The invention provides a method to reduce estrogen receptor promoted replication of a cell. The method involves contacting the cell with a polypeptide or peptidomimetic of the invention in any combination or within a composition. The cell can also be contacted with a composition containing such a polypeptide, peptidomimetic, or both a polypeptide and peptidomimetic. Contact of the polypeptide or peptidomimetic with the cell is thought to inhibit the activity or signaling of endogenous estrogen receptors expressed within the cell undergoing replication. Examples of such replicating cells include breast cancer cells and uterine cancer cells, as well as cells that cause endometriosis.

A cell that is contacted with a polypeptide or peptidomimetic may be contained within a mammal such that it is contacted with the polypepide or peptidomimetic in vivo. For example, a cell may be contacted with a polypepide or peptidomimetic of the invention while included within a human.

Method to increase estrogen receptor activity in a cell

The invention provides a method to increase estrogen receptor activity in a cell. The method involves contacting the cell with an antibody, a peptide aptamer, a composition containing an antibody or peptide aptamer, or any combination thereof. It is thought that contacting a cell with such an antibody or peptide aptamer will block ER-α36 from inhibiting the action of endogenous estrogen receptors and will therefore increase expression of estrogen receptor regulated genes within the cell.

Increasing estrogen receptor activity is thought to reduce or eliminate negative aspects of Alzheimer's disease, Parkinson's disease, multiple sclerosis, depression, bipolar disorder, schizophrenia, osteoporosis, and ischemic stroke.

Method to screen for an agent that promotes or inhibits dimerization of estrogen receptors

The invention provides a method to screen for an agent that promotes or inhibits dimerization of an estrogen receptor. Generally, the method involves contacting a candidate agent with ER-α36 and ER-α46, ER-α66 or ER-β, and determining if the candidate agent promotes or inhibits dimerization of ER-α36 with ER-α46, ER-α66 or ER-β. The method can be conducted in vivo or in vitro.

In one example, a cell in which ER-α36 and ER-α46, ER-α66, ER-β, or any combination thereof are expressed is contacted with a candidate agent. The amount of ER-α36 that forms a dimer with ER-α36, ER-α46, ER-α66 or ER-β is then compared to the amount of ER-α36 that forms a dimer with ER-α36, ER-α46, ER-α66 or ER-β in a cell that was not contacted with the candidate agent. If the actual or relative amount of ER-α36 complexes in the cell contacted with the candidate agent are greater than in the control cell that was not contacted with the candidate agent, then the candidate agent is classified as promoting dimerization of the estrogen receptor. If the actual or relative amount of ER-α36 complexes in the cell contacted with the candidate agent are less than in the control cell that was not contacted with the candidate agent, then the candidate agent is classified as inhibiting dimerization of the estrogen receptor.

The actual or relative amount of a complex containing ER-α36 can be determined by separating ER-α36 containing complexes from a cell lysate through use of an antibody or peptide aptamer that binds to ER-α36. The amount of ER-α46, ER-α66, or ER-β that is separated can also be determined through immunological methods using antibodies that are known to bind to ER-α46, ER-α66, or ER-β. Other immunological based assays known in the art may also be used to practice the method of the invention. The amount of ER-α36, ER-α46, ER-α66, and ER-β in an immunoprecipitated complex can also be readily determined through use of denaturing gel electrophoresis that is conducted according to standard protocols.

In another example, a fusion polypeptide can be used within the method of the invention to determine whether a candidate agent increases or decreases dimerization of an estrogen receptor. In this example, a fusion polypeptide having a carrier polypeptide fused to ER-α36 is expressed within a test cell that is contacted with a candidate agent. The amount of fusion polypeptide that forms a dimer with ER-α36, ER-α46, ER-α66, or ER-β is then compared to the amount of fusion polypeptide that forms a dimer with ER-α36, ER-α46, ER-α66, or ER-β in a cell that was not contacted with the candidate agent. If the actual or relative amount of fusion polypeptide complexes in the cell contacted with the candidate agent are greater than in the control cell that was not contacted with the candidate agent, then the candidate agent is classified as promoting dimerization of the estrogen receptor. If the actual or relative amount of fusion polypeptide complexes in the cell contacted with the candidate agent are less than in the control cell that was not contacted with the candidate agent, then the candidate agent is classified as inhibiting dimerization of the estrogen receptor.

The actual or relative amount of a complex containing a fusion polypeptide can be determined by separating the fusion polypeptide complexes from a cell lysate through use of a material that binds to the carrier protein portion of the fusion polypeptide. For example, antibodies that bind to the carrier protein can be used to separate complexes containing the fusion polypeptide according to standard immunological methods. Numerous antigenic carrier proteins are known in the art and have been reported (Sambrook and Russell, Molecular Cloning, A Laboratory

Manual, Cold Spring Harbour Laboratory Press, 3rd edition, Cold Spring Harbor, New York (2001)). Examples of such antigenic carrier proteins include glutathione-S-transferase, maltose-binding protein, chitin-binding protein, and polypeptides having the following amino acid sequences: QFFGLM (SEQ ID NO:2); EQKLISEEDL (SEQ ID NO:3); KAEDESS (SEQ ID NO:4); YPYDVPDYA (SEQ ID NO:5); DYKDDDDK (SEQ ID NO:6); YTDIEMNRLGK (SEQ ID NO:7); MASMTGGQQMG (SEQ ID NO:8); DTYRYI (SEQ ID NO:9); TDFYLK (SEQ ID NO:10); HHHHHH (SEQ ID NO:11); HPOL (SEQ ID NO:12); QYPALT (SEQ ID NO:13); QRQYGDVFKGD (SEQ ID NO:14); EYMPME (SEQ ID NO:15); EFMPME (SEQ ID NO:16); RYIRS (SEQ ID NO:17), and the like (Sambrook and Russell, Molecular Cloning, A Laboratory Manual, Cold Spring Harbour sLaboratory Press, 3rd edition, Cold Spring Harbor, New York (2001); New England Biolabs, Beverly, MA). Complexes containing a fusion polypeptide can also be separated by contacting a cell lysate with material to which the carrier protein binds. For example, a complex that includes glutathione-S-transferase as a carrier protein can be separated from a cell lysate by applying the lysate to a column on which glutathione is immobilized, and then eluting the complexes from the column. The amount of ER-α36, ER-α46, ER-α66, and ER-β in a separated complex can be readily determined through use of denaturing gel electrophoresis that is conducted according to standard protocols.

The method can also be practiced in vitro. In one example, ER-α36 and ER-α46, ER-α66, or ER-β can be incubated in vitro under conditions known to allow dimerization of the estrogen receptor (Tamrazi et al., Mol. Endocrin., 16:2706 (2002)). The actual or relative amount of complexes containing ER-α36 can be determined through use of immunological methods as described above.

In vivo dimerization of ER-α36 with ER-α36, ER-α46, or ER-α66 on the plasma membrane of a cell can be determined through use of a non-cleavable cell-impermeable cross-linker. In this example, a cross-linker is incubated with cells under conditions where the cross-linker links estrogen receptors that are dimerized on the surface of the cell. The cell can then be lysed and the cross-linked estrogen receptors can be immunoprecipitated and separated using denaturing gel

electrophoresis. The identity of the cross-linked complexes can then be determined through use of ER-α36, ER-α46 and ER-α66 specific antibodies or peptide aptamers. Numerous cross-linking agents, cells, and antibodies are known in the art and are commercially available (Pierce Biotechnology, Inc., Rockford, IL; RDI Research Diagnostics, Inc., Flanders, NJ; ATCC, Manassas, VA).

The method can be used to screen candidate agents for their ability to interfere with the dimerization of ER-α36 with ER-α36, ER-α46, ER-α66 or ER-β by incubating a candidate agent with cells that are cross-linked and analyzing the cross-linked complexes according to the above-described method. The amount of estrogen receptor cross-linking in cells that were contacted with a candidate agent can then be compared to the amount of estrogen receptor cross-linking in corresponding control cells that were not contacted with the candidate agent. An increase in the amount of cross-linked estrogen receptor in cell contacted with the candidate agent relative to cross-linking in the control cells indicates that the candidate agent promotes dimerization of estrogen receptors. A decrease in the amount of cross-linked estrogen receptor in cell contacted with the candidate agent relative to cross-linking in the control cells indicates that the candidate agent inhibits dimerization of estrogen receptors.

In one example, MCF7 (American Type Tissue Culture Collection accession number HTB-22, Manassas, VA), ST1 cells (described herein), or ST3 cells (described herein), can be incubated with 2 mM of BS3 cross-linking agent in phosphate buffered saline (PBS) on ice for 30 minutes. The cells can then be washed extensively with PBS. Cells treated with the BS3 cross-linker can be disrupted, immunoprecipitated with the anti-ER-α antibody H222, and separated on an SDS-PAGE gel. ER-α complexes can then be detected by western blot analysis using an ER-α36 specific antibody. Alternatively, the protein complexes with molecular weights corresponding to homodimers of ER-α36, ER-α46 and ER-α66, and heterodimers thereof can be differentiated with antibodies that recognize different epitopes of ER-α. Examples of such antibodies include H222 that recognizes all three isoforms of ER-α; F-10 that recognizes an N-terminal epitope of ER-α and will therefore only recognize ER-α66; and D-10 that recognizes a C-terminal epitope and

will therefore recognize ER-α66 and ER-α46. The presence of ER-β in these dimers can also be tested using an antibody that recognizes ER-β. The ability of ER-α36 to form homodimers with itself or heterodimers with ER-α46 and ER-α66 on the plasma membrane can be assessed through use of such a method. This method can be conducted in the presence or absence of a candidate agent according to the above-described method to determine if the candidate agent promotes or inhibits dimerization of the estrogen receptor.

Dimerization of ER-α36 with ER-α36, ER-α46, or ER-α66 in the cell nucleus can also be determined in vivo. In one example, an electrophoresis mobility shift assay (EMSA) can be conducted with a DNA probe containing a consensus estrogen response element (ERE) derived from the 5'-flanking region of the chicken apo-VLDL II gene (186 to –156) as a probe. Nuclear extracts from MCF7 cells can be incubated with a ^{32}P labeled probe and separated by non-denaturing electrophoresis on a 4% polyacrylamide gels in 1 X TBE. To determine the specificity of the protein-complexes, cold DNA oligomer containing the wild-type ERE or a mutated ERE sequence can be included in the assays. The identities of the protein-DNA complexes can be ascertained by supershift assays using epitope-specific antibodies and an ER-α36 specific antibody. The anti-ER-β antibody can also be used to identify protein-DNA complexes that contain ER-β. This method can be conducted in the presence or absence of a candidate agent to determine if the candidate agent promotes or inhibits dimerization of the estrogen receptor as described above.

The invention also provides an in vitro assay to determine if a candidate agent promotes or inhibits estrogen receptor dimerization. EMSA can also be performed with estrogen receptors that are produced in vitro using rabbit reticulocyte lysates. The TNT-coupled rabbit reticulocyte system (Promega) programmed with appropriate individual constructs of ER-α isoforms can be used to synthesize homodimers of all three ER-α isoforms. Heterodimers of ER-α isoforms can be produced through use of the TNT system using equimolar concentrations of constructs encoding ER-β, ER-α66, ER-α46, and ER-α36 in different combinations. The products produced in the reticulocyte lysates can be assayed in the presence or absence of a candidate agent to

determine if the candidate agent promotes or inhibits dimerization of estrogen receptors.

In another example, fluorescence resonance energy transfer (FRET) can be used to determine if a candidate agent promotes or inhibits dimerization of an estrogen receptor. Fluorescence resonance energy transfer occurs when an excited fluorescence donor emits light of a wavelength that excites a fluorescence acceptor. The excited fluorescence acceptor then emits light after being excited by the fluorescence donor. The amount of light emitted by the fluorescent acceptor can be detected to determine the distance of the fluorescence donor to the fluorescence acceptor. Accordingly, fluorescence resonance energy transfer can be used to determine if a candidate agent promotes or inhibits estrogen receptor dimerization. For example, ER-α36 can be linked to a fluorescence donor and ER-α66 can be linked to a fluorescence acceptor. A test solution containing a candidate agent, the fluorescence donor linked ER-α36, and the fluorescence acceptor linked ER-α66 is incubated under conditions that allow dimerization of ER-α36 with ER-α66 (Tamrazi et al., Mol. Endocrin., 16:2706 (2002)). The sample solution, or a portion thereof, is analyzed using a fluorometer to determine the actual or relative amount of fluorescence resonance energy transfer from the fluorescence donor to the fluorescence acceptor. The amount of fluorescence transfer can then be compared to the amount of fluorescence transfer in a corresponding control solution that lacks the candidate agent. An increase in fluorescence transfer in a test solution compared to the fluorescence transfer in a corresponding control solution indicates that the candidate agent promotes estrogen receptor dimerization. A decrease in fluorescence transfer in a test solution compared to the fluorescence transfer in a corresponding control solution indicates that the candidate agent inhibits estrogen receptor dimerization.

Numerous donor fluorophores and acceptor fluorophores are known in the art and may be used within the method. Examples of such donor fluorophores include, eukaryotic cyan fluorescent protein, eukaryotic green fluorescent protein, eukaryotic yellow fluorescent protein, cy3, cy3.5, fluorescein, IAEDANS, EDANS, BODIPY FL, Fluorescein QSY 7, and the like. Examples of such acceptor fluorophores include,

eukaryotic cyan fluorescent protein, eukaryotic green fluorescent protein, eukaryotic yellow fluorescent protein, cy3, cy3.5, tetramethylrhodamine, fluorescein, dabcyl, BODIPY FL, QSY 9 dyes, and the like. Numerous fluorophores and methods to link them are known in the art and are commercially available (Sambrook and Russell, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, 3rd edition, Cold Spring Harbor, New York (2001); Amersham Pharmacia Biotech, Piscataway, NJ, USA; Molecular Probes, Inc., Eugene, OR).

Fluorescence quenching may also be used to identify agents that promote or inhibit estrogen receptor dimerization. In this example, one monomer of the estrogen receptor dimer is linked to a fluorescence donor, and the other monomer of the estrogen receptor is dimer is linked to a fluorescence quencher. Dimerization of the two monomers causes fluorescence transmission from the fluorescence donor to be quenched by the fluorescence quencher. Accordingly, ER-α36, ER-α46, ER-α66, or ER-β can be linked to either a fluorescence donor or a fluorescence quencher. For example, a test solution containing a candidate agent, a fluorescence donor linked ER-α36, and a fluorescence quencher linked ER-α36, ER-α46, ER-α66, or ER-β is incubated under conditions that allow dimerization of ER-α36 with ER-α36, ER-α46, ER-α66, or ER-β (Tamrazi et al., Mol. Endocrin., 16:2706 (2002)). The sample solution, or a portion thereof, is analyzed using a fluorometer to determine the actual or relative amount of fluorescence quenching, or fluorescence transmission, in the test solution. The amount of fluorescence quenching, or fluorescence transmission, in the test solution is then compared to the amount of fluorescence quenching or transmission, in a corresponding control solution that lacks the candidate agent. An increase in fluorescence quenching, or a decrease in fluorescent transmission, in the test solution compared to the corresponding control solution indicates that the candidate agent promotes estrogen receptor dimerization. A decrease in fluorescence quenching, or an increase is fluorescence transmission, in the test solution compared to the fluorescence transfer in a corresponding control solution indicates that the candidate agent inhibits estrogen receptor dimerization. Numerous fluorescence donors and fluorescence quenchers are known in the art and may be used within the method (Sambrook and Russell, Molecular Cloning, A Laboratory Manual, Cold

Spring Harbour sLaboratory Press, 3rd edition, Cold Spring Harbor, New York (2001); Amersham Pharmacia Biotech, Piscataway, NJ, USA; Molecular Probes, Inc., Eugene, OR).

5'-flanking region of ER-α36 that contains an ER-α36 promoter

The invention provides an ER-α36 5'-promoter region. The ER-α36 can include and Estrogen Response Element (ERE). The 5'-promoter region of ER-α36 is a polynucleotide having a nucleotide sequence that facilitates expression of an operably linked open reading frame, such an open reading frame that encodes the ER-α36 estrogen receptor isoform. In one example, an ER-α36 promoter of the invention has a nucleotide sequence corresponding to SEQ ID NO:22. In another example, an ER-α36 promoter has at least 70% sequence identity to the nucleotide sequence of SEQ ID NO:22 and is able to promote expression of an operably linked open reading frame in an estrogen dependent manner. Preferably the ER-α36 promoter is a polynucleotide having at least 70% nucleotide identity to SEQ ID NO:22. More preferably the ER-α36 promoter has a nucleotide sequence that is at least single unit percentages greater than 70% identical to SEQ ID NO:22, for example 71%, 72%, 73% identity, and so on to 100% identity to SEQ ID NO:22. Even more preferably, the ER-α36 promoter has a nucleotide sequence that is at least 80% identical to SEQ ID NO:22. Still even more preferably, the ER-α36 promoter has a nucleotide sequence that is at least 90% identical to SEQ ID NO:22. Yet still even more preferably, the ER-α36 promoter has a nucleotide sequence that is at least 95% identity to SEQ ID NO:22. Most preferably, the ER-α36 promoter has a nucleotide sequence that is 100% identical to SEQ ID NO:22.

Polynucleotides can be tested for their ability to promote the expression of an operably linked open reading frame in an estrogen dependent manner by inserting the polynucleotide into a vector such that it is operably linked to an open reading frame that encodes a detectable marker. The recombinant vector can then be transformed into a cell that expresses estrogen receptors to produce a transformed cell. The transformed cell can then be contacted with estrogen and tested for estrogen promoted expression of the detectable marker. An ER-α36 estrogen response element may be

operably linked to an open reading frame alone, or in combination with other regulatory elements. Such regulatory elements include, promoters, repressor binding sites, introns, enhancers, termination signals, and the like. Regulatory elements can be operably linked to the 5' or 3' end of an open reading frame.

An open reading frame can encode numerous detectable markers. Examples of such detectable markers include, but are not limited to, fluorescent proteins, enzymes, antigenic markers, and the like.

An open reading frame can encode numerous selectable markers. Examples of selectable markers include, but are not limited to, markers that confer drug resistance or drug susceptibility, markers that either cause or correct a nutritional deficiency, and the like.

An ER-α36 estrogen response element may be utilized within the methods described herein to identify agents that promote or inhibit estrogen dependent gene expression. Alternatively, a nucleic acid construct may be constructed that contains a vector and an ER-α36 estrogen response element operably linked to an open reading frame that confers a useful characteristic onto a cell. The nucleic acid construct can then be transformed into a cell through use of standard procedures to produce a transformed cell wherein the open reading frame is expressed in an estrogen dependent manner. Such cells may be used for the production of useful gene products.

Method to identify an agent that increases or decreases ER-α36 promoter activity

The invention provides a method to identify an agent that increases or decreases ER-α36 expression. In one example, the method involves contacting a test cell that contains a nucleic acid construct having an ER-α36 promoter operably linked to an open reading frame with a candidate agent, and determining if the candidate agent causes an increase or decrease in the expression of the open reading frame compared to expression of the open reading frame in a corresponding control cell that was not contacted with the candidate agent. The method of the invention can be readily configured into a high-throughput screening format according to procedures commonly used within the pharmaceutical industry.

Virtually any candidate agent can be screened for the ability to increase or decrease ER-α36 expression, or increase or decrease ER-α36 promoter activity. Examples of potential candidate agents include chemicals, peptides, sugars, carbohydrates, lipids and the like. Sources for potential agents to be screened include, for example, chemical compound libraries; fermentation media of bacteria, fungi, yeast, eukaryotic cells, and the like; and cell extracts of plants and other vegetations.

Numerous types of prokaryotic and eukaryotic cells may be used within the method of the invention. Examples of such cells include, but are not limited to, *E. coli* cells, *Salmonella* cells, yeast cells, mouse cells, hamster cells, and human cells that are exemplified by mammary and uterine cells.

The open reading frame can encode nearly any detectable product. For example, the open reading frame can encode an RNA that can be detected through methods known in the art, such as nuclease protection assays, hybridization assays, and the like. The RNA can also exhibit a detectable activity, such as a small interfering RNA, an anti-sense RNA, or a ribozyme. The open reading frame can also encode a detectable marker, such as a polypeptide. Examples of detectable polypeptides include fluorescent proteins, antigenic proteins, enzymes, toxins, and the like.

Method to identify an ER-α36 ligand

The invention provides a method to identify a ligand that is bound by ER-α36. In one example, an ER-α36 fusion polypeptide is created that has an ER-α36 ligand binding domain fused to the AF2 domain and the estrogen responsive element binding domain from ER-α66. Examples of fusion polypeptides that include an estrogen receptor ligand binding domain, and methods for their use, have been reported (Kametaka et al., Cancer Sci., 94:639 (2003); Zhu et al., World J. Gastroenterol., 9:1844 (2003); Chen et al., Oncogene, published on-line Dec 15). A cell can be transformed with a nucleic acid construct that encodes an ER-α36 fusion polypeptide so that the cell expresses the fusion polypeptide. The cell can also be transformed with a nucleic acid construct having an estrogen response element operably linked to an open reading frame that encodes a detectable marker. Numerous examples of

detectable markers are known in the art and are disclosed herein. Candidate ligands can then be screened for their ability to bind, and activate, the ER-α36 fusion polypeptide by determining if the candidate ligand promotes expression of the detectable marker. Cells that do not endogenously express estrogen receptors are preferred for use in the method. However, cells that express an endogenous estrogen receptor can also be used within the method by determining if the candidate ER-α36 ligand promotes increased expression of the detectable marker relative to expression of the detectable marker in a corresponding control cell that was not contacted with the candidate ligand. Examples of cells that can be used within a method of the invention include bacteria, yeast, mammalian cells, and the like.

Numerous ER-α36 fusion polypeptides can be created that include the ligand binding domain from ER-α36 fused to a DNA-binding and transcription regulatory domain from a transcription factor or other DNA-binding protein. For example, an ER-α36 fusion polypeptide can be created that has a polypeptide of SEQ ID NO:20 fused to a DNA-binding and transcription regulatory domain from a transcription factor. These fusion polypeptides can be used to promote expression of a detectable marker, or inhibit expression of a detectable marker through use of nucleic acid constructs containing a binding element. Additionally, fusion polypeptides can be created that induce apoptosis of a cell in a ligand dependent manner providing for rapid screening of candidate ligands (Kametaka et al., Cancer Sci., 94:639 (2003)).

Guidance regarding the structure of ligands that are bound by ER-α36 is provided by the structure of known ligands for the estrogen receptor and by the structure of 4-estren-3α,17β-diol, a synthetic compound that does not have classical transcriptional activity (Innovative Research of America, Sarasota, FL). Accordingly, the structure of known estrogen receptor ligands or 4-estren-3α,17β-diol can be modified and used as a candidate ligand to define structural features that are recognized by ER-α36.

It is thought that ligands that are bound by ER-α36 are useful for treating osteoporosis. It has been shown that sex steroids protect the adult murine skeletal system through a mechanism that is distinct from that used to preserve the mass and function of reproductive organs (Kousteni et al., Science, 298:843 (2002)). In

contrast, the classical genotropic actions of sex steroid receptors are dispensable for their bone protective effects, but essential for their effects on reproductive tissues. The synthetic ligand, 4-estren-3α,17β-diol (estrin), reproduces the nongenotropic effects of sex steroids without affecting classical transcription, and increases bone mass and strength in ovariectomized females without affecting reproductive organs. These results suggest that separate pathways may act to regulate reproductive tissues and bone tissue.

Two genetic polymorphisms have been correlated to bone loss in women suffering from osteoporosis. These polymorphisms are the Pvu II and the Xba I polymorphisms. Based on the results described herein, it is thought that these polymorphisms cause a down-regulation in ER-α36 expression. Therefore, decreased ER-α36 activity is thought to be correlated with bone loss in patients suffering from osteoporosis. Accordingly, ligands that are bound by ER-α36 are thought to be useful for activating the activity of ER-α36 and promote increases in bone mass and strength.

Kits

The invention provides kits that contain reagents used for determining if a cell is refractory to treatment with tamoxifen. Such kits can contain packaging material, and an antibody, peptide aptamer, or both an antibody and peptide aptamer that binds to the ER-α36 estrogen receptor isoform. Such kits may also be used by medical personal for the formulation of compositions, such as pharmaceutical compositions, that contain an antibody or peptide aptamer of the invention.

The packaging material will provide a protected environment for the antibody or peptide aptamer. For example, the packaging material may keep the antibody or peptide aptamer from being contaminated. In addition, the packaging material may keep an antibody or peptide aptamer in solution from becoming dry. Examples of suitable materials that can be used for packaging materials include glass, plastic, metal, and the like. Such materials may be silanized to avoid adhesion of an antibody or peptide aptamer to the packaging material.

In one example, the invention provides a kit that includes packaging material, a first antibody that specifically binds to the ER-α36 estrogen receptor isoform, and a

second antibody that specifically binds to the ER-α66 estrogen receptor isoform. The kit may optionally include additional components such as buffers, reaction vessels, secondary antibodies, and syringes. In one example, a kit can include a first antibody that specifically binds to the ER-α36 estrogen receptor isoform, a second antibody that specifically binds to the ER-α66 estrogen receptor isoform, a syringe, a tray to which the first or second antibody can be immobilized, wash buffer, and packaging material.

In another example, the invention provides a kit that includes a first antibody that specifically binds to the ER-α36 estrogen receptor isoform, a second antibody that specifically binds to an ER-α66 estrogen receptor isoform, and packaging material. The kit may optionally include additional components such as buffers, reaction vessels, secondary antibodies, and syringes.

Table I

Amino acid and nucleotide sequences

SEQ ID NO	Description	Amino acid and nucleotide Sequences
18	ER-α66 Accession Numbers M12674 AAA52399	MTMTLHTKASGMALLHQIQGNELEPLNRPQLKIPLERPLGE VYLDSSKPAVYNYPEGAAYEFNAAAAANAQVYGQTGLPYGP GSEAAAFGSNGLGGFPPLNSVSPSPLMLLHPPPQLSPFLQP HGQQVPYYLENEPSGYTVREAGPPAFYRPNSDNRRQGGRER LASTNDKGSMAMESAKETRYCAVCNDYASGYHYGVWSCEGC KAFFKRSIQGHNDYMCPATNQCTIDKNRRKSCQACRLRKCY EVGMMKGGIRKDRRGGRMLKHKRQRDDGEGRGEVGSAGDMR AANLWPSPLMIKRSKKNSLALSLTADQMVSALLDAEPPILY SEYDPTRPFSEASMMGLLTNLADRELVHMINWAKRVPGFVD LTLHDQVHLLECAWLEILMIGLVWRSMEHPVKLLFAPNLLL DRNQGKCVEGMVEIFDMLLATSSRFRMMNLQGEEFVCLKSI ILLNSGVYTFLSSTLKSLEEKDHIHRVLDKITDTLIHLMAK AGLTLQQQHQRLAQLLLILSHIRHMSNKGMEHLYSMKCKNV VPLYDLLLEMLDAHRLHAPTSRGGASVEETDQSHLATAGST SSHSLQKYYITGEAEGFPATV

19	ER-α66 Accession Number M12674 AY425004	ATGACCATGACCCTCCACACCAAAGCATCTGGGATGGCCCTACTGCATCAGAT CCAAGGGAACGAGCTGGAGCCCCTGAACCGTCCGCAGCTCAAGATCCCCCTGG AGCGGCCCCTGGGCGAGGTGTACCTGGACAGCAGCAAGCCCGCCGTGTACAAC TACCCCGAGGGCGCCGCCTACGAGTTCAACGCCGCGGCCGCCGCCAACGCGCA GGTCTACGGTCAGACCGGCCTCCCCTACGGCCCCGGGTCTGAGGCTGCGGCGT TCGGCTCCAACGGCCTGGGGGGTTTCCCCCCACTCAACAGCGTGTCTCCGAGC CCGCTGATGCTACTGCACCCGCCGCCGCAGCTGTCGCCTTTCCTGCAGCCCCA CGGCCAGCAGGTGCCCTACTACCTGGAGAACGAGCCCAGCGGCTACACGGTGC GCGAGGCCGGCCCGCCGGCATTCTACAGGCCAAATTCAGATAATCGACGCCAG GGTGGCAGAGAAAGATTGGCCAGTACCAATGACAAGGGAAGTATGGCTATGGA ATCTGCCAAGGAGACTCGCTACTGTGCAGTGTGCAATGACTATGCTTCAGGCT ACCATTATGGAGTCTGGTCCTGTGAGGGCTGCAAGGCCTTCTTCAAGAGAAGT ATTCAAGGACATAACGACTATATGTGTCCAGCCACCAACCAGTGCACCATTGA TAAAAACAGGAGGAAGAGCTGCCAGGCCTGCCGGCTCCGCAAATGCTACGAAG TGGGAATGATGAAAGGTGGGATACGAAAAGACCGAAGAGGAGGGAGAATGTTG AAACACAAGCGCCAGAGAGATGATGGGGAGGGCAGGGGTGAAGTGGGGTCTGC TGGAGACATGAGAGCTGCCAACCTTTGGCCAAGCCCGCTCATGATCAAACGCT CTAAGAAGAACAGCCTGGCCTTGTCCCTGACGGCCGACCAGATGGTCAGTGCC TTGTTGGATGCTGAGCCCCCCATACTCTATTCCGAGTATGATCCTACCAGACC CTTCAGTGAAGCTTCGATGATGGGCTTACTGACCAACCTGGCAGACAGGGAGC TGGTTCACATGATCAACTGGGCGAAGAGGGTGCCAGGCTTTGTGGATTTGACC CTCCATGATCAGGTCCACCTTCTAGAATGTGCCTGGCTAGAGATCCTGATGAT TGGTCTCGTCTGGCGCTCCATGGAGCACCCAGTGAAGCTACTGTTTGCTCCTA ACTTGCTCTTGGACAGGAACCAGGGAAAATGTGTAGAGGGCATGGTGGAGATC TTCGACATGCTGCTGGCTACATCATCTCGGTTCCGCATGATGAATCTGCAGGG AGAGGAGTTTGTGTGCCTCAAATCTATTATTTTGCTTAATTCTGGAGTGTACA CATTTCTGTCCAGCACCCTGAAGTCTCTGGAAGAGAAGGACCATATCCACCGA GTCCTGGACAAGATCACAGACACTTTGATCCACCTGATGGCCAAGGCAGGCCT GACCCTGCAGCAGCAGCACCAGCGGCTGGCCCAGCTCCTCCTCATCCTCTCCC ACATCAGGCACATGAGTAACAAAGGCATGGAGCATCTGTACAGCATGAAGTGC AAGAACGTGGTGCCCCTCTATGACCTGCTGCTGGAGATGCTGGACGCCCACCG CCTACATGCGCCCACTAGCCGTGGAGGGGCATCCGTGGAGGAGACGGACCAAA GCCACTTGGCCACTGCGGGCTCTACTTCATCGCATTCCTTGCAAAAGTATTAC ATCACGGGGGAGGCAGAGGGTTTCCCTGCCACAGTCTGA
20	ER-α36 Accession Number CAE45969.1	MAMESAKETRYCAVCNDYASGYHYGVWSCEGCKAFFKRSIQGHNDYMCPATNQ CTIDKNRRKSCQACRLRKCYEVGMMKGGIRKDRRGGRMLKHKRQRDDGEGRGE VGSAGDMRAANLWPSPLMIKRSKKNSLALSLTADQMVSALLDAEPPILYSEYD PTRPFSEASMMGLLTNLADRELVHMINWAKRVPGFVDLTLHDQVHLLECAWLE ILMIGLVWRSMEHPGKLLFAPNLLLDRNQGKCVEGMVEIFDMLLATSSRFRMM NLQGEEFVCLKSILLLNSGISHVEAKKRILNLHPKIFGNKWFPRV

21	ER-α36 Accession Number BX640939	ATGGCTATGGAATCTGCCAAGGAGACTCGCTACTGTGCAGTGTGCAATGACTA TGCTTCAGGCTACCATTATGGAGTCTGGTCCTGTGAGGGCTGCAAGGCCTTCT TCAAGAGAAGTATTCAAGGACATAACGACTATATGTGTCCAGCCACCAACCAG TGCACCATTGATAAAAACAGGAGGAAGAGCTGCCAGGCCTGCCGGCTCCGCAA ATGCTACGAAGTGGGAATGATGAAAGGTGGGATACGAAAAGACCGAAGAGGAG GGAGAATGTTGAAACACAAGCGCCAGAGAGATGATGGGGAGGGCAGGGGTGAA GTGGGGTCTGCTGGAGACATGAGAGCTGCCAACCTTTGGCCAAGCCCGCTCAT GATCAAACGCTCTAAGAAGAACAGCCTGGCCTTGTCCCTGACGGCCGACCAGA TGGTCAGTGCCTTGTTGGATGCTGAGCCCCCCATACTCTATTCCGAGTATGAT CCTACCAGACCCTTCAGTGAAGCTTCGATGATGGGCTTACTGACCAACCTGGC AGACAGGGAGCTGGTTCACATGATCAACTGGGCGAAGAGGGTGCCAGGCTTTG TGGATTTGACCCTCCATGATCAGGTCCACCTTCTAGAATGTGCCTGGCTAGAG ATCCTGATGATTGGTCTCGTCTGGCGCTCCATGGAGCACCCAGGGAAGCTACT GTTTGCTCCTAACTTGCTCTTGGACAGGAACCAGGGAAAATGTGTAGAGGGCA TGGTGGAGATCTTCGACATGCTGCTGGCTACATCATCTCGGTTCCGCATGATG AATCTGCAGGGAGAGGAGTTTGTGTGCCTCAAATCTATTCTTTTGCTTAATTC TGGTATCTCACATGTAGAAGCAAAGAAGAGAATCCTGAACTTGCATCCTAAAA TATTTGGAAACAAGTGGTTTCCTCGTGTCTAA
22	ER-α36 5'-flanking sequence	GGTACCCGCGCCCGCGCCGCCCGTCGGGGTGGCCGCCGCGC CCGGCAGGAGGGAGGGAGGGAGGGAGGGAGAAGGGAGAGCC TAGGGAGCTGCGGGAGCCGCGGGACGCGCGACCCGAGGGTG CGCGCAGGGAGCCCGGGGCGCGCGGCCCAGCCCGGGGGTTC TGCGTGCAGCCCGCGCTGCGTTCAGAGTCAAGTTCTCTCGC CGGGCAGCTGAAAAAAACGTACTCTCCACCCACTTACCGTC CGTGCGAGAGGCAGACCCGAAAGCCCGGGCTTCCTAACAAA ACACACGTTGGAAAACCAGACAAAGCAGCAGTTATTTGTGG GGGAAAACACCTCCAGGCAAATAAACACGGGGCGCTTTGAG TCACTTGGGAAGGTCTCGCTCTTGGCATTTAAAGTTGGGGG TGTTTGGAGTTAGCAGAGCTCAGCAGAGTTTTATTTATCCT TTTAATGTTTTTGTTTAATGTGCTCCCCAAATTTCCTTTCA TCTAGACTATTTGATTGGAAATATGTCAGCTATGATGATGA CTTTCTGGGAAGCGATTCCTGTCACCCGCTTTCCCCTCCTC CCCACCCCACGTCCTGGGGCTTTAGAGAGCGATTGGGAGTT GAATGGGTCTGATTTCGGAGTTAGCTGGCTGAGTCCGCGCT GGAGCGGATTGCTGGCATGTGACTTCTGACAGCCGGAAATT TGTAGGTGTCCCGCGAGTTTAAAACAAGCCATATGGAAGCA CAAGTGCTTAAAAA
1	C-terminal 27 amino acids of ER-α36	GISHVEAKKRILNLHPKIFGNKWFPRV

The present invention is illustrated by the following examples. It is to be understood that the particular examples, materials, amounts, and procedures are to be interpreted broadly in accordance with the scope and spirit of the invention as set forth herein.

EXAMPLES

Example I

Caveolin-1 haploinsufficiency produces activation of ER-α expression and estrogen stimulated transformation of normal breast epithelial cells

A gene-trapped library of cell clones from normal human mammary epithelial MCF10A cells was prepared through use of a poly-A trap retrovirus vector (RET) obtained from Dr. Philip Leder's laboratory at Harvard Medical School (Ishida et. al., Nucl. Acid Res., 27:580 (1999)). Briefly, this vector used an improved poly-A trap strategy for the efficient identification of functional genes regardless of their expression status in target cells. A combination of a strong splice acceptor and an effective polyadenylation signal assures the complete disruption of the function of "trapped" genes. Inclusion of a promoterless GFP cDNA in the RET vector allows the expression pattern of the trapped gene to be easily monitored in living cells. A retrovirus containing the RET vector was used to infect MCF10A cells. The cells were then screened for G418-resistant to establish a gene-trapped library of MCF10A cells. After selection by G418 for three weeks, GFP expression, under the control of the endogenous promoter of the "trapped' gene, was monitored and G418 resistant and GFP expressing clones were then selected. This library represented $3x10^5$ independent infected clones in which one allele of a functional gene was disrupted by the RET vector.

It was thought that loss of expression of genes with tumor suppression activity could confer the transformed phenotype to normal MCF10A cells. A soft-agar cloning assay was performed and cells from the gene–trapped cell library that acquired anchorage-independent growth, a characteristic of the transformed phenotype, were identified. More than 100 positive colonies (≥30 cells) from the library of G418-resistant cells grew in soft-agar while the parental MCF10A cells did not. Twenty cell clones were isolated, expanded, and then selected again in soft-agar containing regular serum plus 10-8 M 17b-estradiol (E2) and dextrin coated charcoal-stripped serum that lacks steroid hormones for three weeks. Four cell clones (ST1, ST3, ST4 and ST6)

that exhibited accelerated growth in soft-agar containing extra E2 were isolated and expanded (Figure 3).

3'-RACE, which permits the capture of unknown 3'mRNA sequences that lie between the exon of a candidate gene and the poly-A tail, was used to clone potential genes whose disruption leads to MCF10A cell transformation. Transformation of the MCF10A cells was thought to be due to positive estrogen signaling. The purified PCR fragments resulting from the RACE procedure were cloned and sequenced. Using a BLASTN search, the DNA sequences from two clones (ST1 and ST3) were matched identically to the sequence of caveolin-1 (Cav-1) exon-3 located on chromosome 7 (GenBank accession number XM048940). This result indicated that an allele of the Cav-1 gene was disrupted in at least 2 clones. In addition to Cav-1, two other genes were identified using the same technique. The gene disrupted in clone ST4 was SPRR1B (GenBank accession number NT-004441.5), a member of the cornifin/small proline-rich protein family involved in structural organization of cornified cell envelopes. Another gene from clone ST6 is a putative novel gene (GenBank accession number 6599139) with no known function.

Cav-1 protein levels were analyzed in parental MCF10A cells to test whether expression levels of Cav-1 were decreased in the cav-1 gene trapped cells. Four cell clones (ST1, 3, 4 and 6) described above, and MCF10A-Ha-ras, (MCF10A cells transformed by a Ha-ras mutant) were analyzed. Compared to the levels in parental MCF10A cells, Cav-1 protein levels were about 2-fold lower in all transformed cells as demonstrated by Western blot analysis (Figure 4). This data is consistent with Cav-1 expression being decreased when only one functional allele of the cav-1 gene is functional in ST1 and ST3 cells. This indicates that Cav-1 haploinsufficiency created by "gene trapping" leads to transformation stimulated by E2. This data also indicates that downregulation of Cav-1 is also involved in transformation resulted from the disruption of other genes in ST4 and ST6 cells.

To determine the mechanism by which Cav-1 haploinsufficiency leads to estrogen-stimulated cell growth and transformation, the expression levels of ER-α and ER-β in the transformed cells described above was examined. It was found that all of the four transformed cell clones expressed ER-α at a level comparable to that in the

Ha-ras transformed cells, whereas parental MCF10A cells and HBL-100 cells, another normal mammary epithelial cell line, expressed undetectable levels of ER-α (Figure 5). ER-β expression was without any change in all of the cells tested (Figure 5). This data indicated that ER-α expression was activated and that estrogen signaling in these transformed cells is responsible for the estrogen-stimulated cell growth on soft-agar. It has been shown before that both ER-α expression and estrogen signaling are activated in Ha-ras transformed cells (Shekhar et. al., Int. J. Oncol., 13:907 (1998) and Shekhar et. al., Am. J. of Pathl., 152:1129 (1998)). The present results indicate that the Ras/MAPK pathway is involved in the regulation of ER-α expression and positive estrogen signaling.

Activation of the MAPK pathway in these transformed cells was analyzed by examining the phosphorylation levels of ERK1/2 using phospho-specific antibodies. It was found that ERK1/2 are highly and constitutively phosphorylated in all transformed cells but not in MCF10A cells (Figure 6).

Taken together, these data indicate that the Cav-1/Ras/MAPK pathway is involved in the activation of ER-α expression during human breast cancer development and cooperates with estrogen signaling pathway to stimulate transformed cell proliferation.

Example II

Identification, cloning, expression and characterization of an isoform of estrogen receptor alpha (ER-α36)

During the course of the work described above, three protein bands (66-kDa, 46-kDa and 36-kDa) were consistently observed in western blot analysis using the Rat anti-ER-α antibody (clone H222) from Research Diagnostic, INC. The H222 antibody recognizes the ligand-binding domain of ER-α. To exclude the possibility that 46-kDa and 36-kDa protein bands were the degradation products of ER-α66, as suggested by a previous report (Abbondanza et. al., Steroids, 58:4 (1993)), cells were lysed in culture plates using a buffer containing 8 M urea and tested by western blot analysis. Three distinct bands were readily observed in Cav-1 haploinsufficient cells,

ST1 and ST3, and MCF7 breast cancer cells (Figure 7). These results indicated the existence of ER-α isoforms that share a similar epitope that is recognized by the antibody H222.

Through a literature search, it was found that a 46-kDa isoform of ER-α had been cloned that functions as a dominant-negative inhibitor of transactivation mediated by the AF-1 domain of ER-α66 (Flouriot et. al., EMBO J., 19:4688 (2000)). A continued search identified a clone from a normal human edometrium cDNA library (RZPD clone number: DKFZp686N23123) that contains a 5.4 kb cDNA. This cDNA clone harbors a 310 amino acid open reading frame that theoretically can produce a protein with a predicted molecular weight of 35.7 kDa. The cDNA sequence of the open-reading frame matched 100% to DNA sequence of the exons 2 to 6 of the ER-α66 gene. The 5'untraslated region (5'UTR) of the cDNA showed 100% homology to the DNA sequence of the first intron of the ER-α66 gene from 734 to 907 (the first base pair of the 34,233 bp first intron of ER-α66 gene was designated as 1). Thus, it was determined that the transcript of ER-α36 is initiated from a previously unidentified promoter in the first intron of the ER-α66 gene.

A small, non-coding novel exon from 734 to 907 of the first intron of the ER-α66 gene was designated as "exon 1'". The exon 1' is then spliced directly into the exon 2 of the ER-α66 gene and continues from exon 2 to exon 6 of the ER-α66 gene. Exon 6 is then spliced to an exon located 64,141 bp downstream of the ER-α66 gene (GeneBank accession number AY425004). The cDNA sequence encoding the last 27 amino acids and the 4,293 bp 3'untranslated region was matched 100% to a continuous sequence from the genomic sequence of clone RP1-1304 on chromosome 6q24.2-25.3 (GeneBank accession number AL78582), indicating the remaining cDNA sequence of this novel ER-α isoform is transcribed from one big exon of 4,374 bp located downstream of the previously reported ER-α66 gene. This exon is thus designated as exon 9 to reflect the extra exon beyond the previous reported eight exons (Figure 8). All of these splicing events are supported by the identification of perfect splice donors and acceptors at the splice juncture. The protein ER-α36 can be produced from a perfect Kozak sequence located in the second exon, the same

initiation codon used to produce ER-α46 (Flouriot et. al., EMBO J., 19:4688 (2000)). ER-α36 differs from ER-α66 by lacking both transcriptional activation domains, AF-1 and AF-2, but retaining the dimerization, DNA-binding and partial ligand-binding domains. It also possesses an extra, unique 27 amino acid domain to replace the last 138 amino acids encoded by exon 7 and 8 of the ER-α66 (Figure 1). Here, this novel isoform of ER-α is herein named ER-α36.

The open reading frame encoding ER-α36 was obtained by using the PCR from the Marathon Ready cDNA prepared from human placenta RNA (Clonetech) according to the procedure described by the manufacture. The PCR primer pairs are designed according to the cDNA sequence of DKFZp686N23123. The 5' primer is 5'-CGGAATTCCGAAGGGAAGTATGGCTATGGAATCC-3' (SEQ ID NO:23) with an EcoRI site at the end, and the 3' primer is 5'-CGGGATCCAGAGGCTTTAGACACGAGGAAAC-3' (SEQ ID NO:24) with a BamHI site at the end. The PCR product was subjected to electrophoresis on a 1% agarose gel, and an expected 1.1 kb DNA fragment was observed (Figure 9). The DNA fragment was purified, digested with EcoRI and BamHI, cloned into a pBluescript vector (pBS- ER-α36) and fully sequenced. The sequence showed 100% identity to the cDNA clone DKFZp686N23123, indicated that ER-α36 is a naturally occurring isoform of ER-α that can be cloned from another source. The predicted amino acid sequence encoded by the open-reading frame is shown in Figure 10.

Transient transfection assays were performed in human embryonic kidney 293 cells using expression vectors containing ER-α66, ER-α46 and ER-α36 to test whether the cloned cDNA will produce the ER-α36 protein. Whole cell extracts from these transfected cells and MCF7 cells were subjected to western blot analysis with the monoclonal antibody H222 raised against the ligand-binding domain of ER-α (Abbondanza et. al., Steroids, 58:4 (1993)). A 36 kDa protein that was recognized by the H222 antibody was produced in ER-α36 vector transfected cells (Figure 11). The size of this protein and its failure to react with the antibody H226 directed to the B-domain of the ER-α66, and with the antibody HC 20 which recognizes the C-terminal

of ER-α66, indicates that the ER-α isoform lacked both the N-terminus and C-terminus of ER-α66, resulting in an ER-α lacking both AF-1 and AF–2 domains.

A series of computer searches were performed on the ER-α36 protein. FindMod and SCANPROSITE algorithms predicted three myristoylation sites in ER-α36, suggesting that it may localize in the peripheral membrane. This is in agreement with the k-nearest neighbors (PSPORT II) algorithm that predicts 21.7%, 34.8%, 17.4%, and 26% of ER-α36 is localized to the nucleus, cytoplasm, mitochondria, and membrane fractions, respectively. This is similar to the prediction for ER-α46 (26.1%, 30.4%, 17.4%, and 26.1%, respectively). By contrast, 73.9% 8.7%, 0.1% and 17.3% of ER-α66 carries comparative predictions. Thus, the differential compartmentalization of ER-α66, ER-α46, and ER-α36 indicates that the functional site and primary role of each receptor may be different.

A computer search was also performed on the putative 5' flanking region of the gene encoding ER-α36 that is located in the first intron of ER-α66 gene. A TATA binding protein (TBP) recognition sequence was found upstream of the cDNA start site and several Sp1, NF-κB and Ap1 binding sites in the 5' flanking region (Figure 12). A perfect half estrogen response element (ERE) site was identified at the 5' upstream region of ER-α36, indicating that ER-α36 is subjected to E2-mediated transcriptional regulation.

All publications, patents and patent applications are incorporated herein by reference. While in the foregoing specification this invention has been described in relation to certain preferred embodiments thereof, and many details have been set forth for purposes of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments and that certain of the details described herein may be varied considerably without departing from the basic principles of the invention.

A Novel Variant of Estrogen Receptor-Alpha is Expressed in ER-negative Breast Cancer and Mediates Membrane-Initiated Estrogen Signaling

Since 1977, numerous studies have indicated the existence of a putative membrane based estrogen receptor. However, the identity of this membrane-based estrogen receptor has not been established. Through an extensive homology search in Gene Bank, we recently identified and cloned a novel variant of ER-α with a predicted molecular weight of 35.7 kDa. The transcript of this ER-α variant is initiated from a previously unidentified promoter in the first intron of the original ER-α gene. This ER-α variant differs from the original ER-α by lacking both transcriptional activation domains (AF-1 and AF-2) but retaining the dimerization, DNA-binding and partial ligand-binding domains. Its ligand-binding domain also possesses a unique 27 amino acid stretch that replaces the last 138 amino acids of the ER-α. In transient transfection assays, this ER-α variant exhibited no intrinsic transcriptional activity but strongly inhibited ligand-dependent and –independent transactivation activities mediated by the original ER-α and ER-β. Immunodetection and subcellular fractionation assays demonstrated that this ER-α variant is expressed on the plasma membrane of mammary epithelial cells, and also in the cytoplasm and nucleus. In cells that express this ER-α variant, 17β-estradiol (E2) treatment activated a membrane-initiated effect of estrogen signaling by transiently activating the mitogen-activated protein kinase (MAPK) pathway that leads to cell proliferation. Antiestrogens such as tamoxifen and ICI 182,780, however, strongly and persistently activated the MAPK pathway that then resulted in the activation of the SAPK/JNK pathway. Immunohistochemistry analysis using a specific antibody aganst this ER-α variant revealed that 26 of 38 cases (68%) breast cancer specimens examined express this ER-α variant on the plasma membrane and cytoplasm, and 12 out of 13 ER-negative breast caner specimens also express this ER-α variant. RT-PCR analysis further showed that this ER-α variant has an inverse expression pattern of the original ER-α in established breast cancer cells. Our findings demonstrate a physiologically important ER-α variant that is involved in membrane-initiated estrogen signaling and suggest that aberrant expression of this ER-α variant contributes to development of ER-positive and-negative human breast cancer.

WHAT IS CLAIMED IS:

1. A method to determine if a test cell responds to, or is refractory to, treatment with a therapeutic agent comprising:

determining a ratio of ER-α36 to ER-α66, ER-α46 or ER-β in the test cell, and

comparing the ratio determined in the test cell to a ratio of ER-α36 to ER-α66, ER-α46 or ER-β in a control cell.

2. The method according to claim 33, wherein the control cell is not refractory to treatment with the therapeutic agent.

3. The method according to claim 33, wherein the control cell is refractory to treatment with the therapeutic agent.

4. The method according to claim 33, wherein the therapeutic agent is centchroman, delmadinone acetate, tamoxifen, droloxifene, idoxifene, raloxifene, toremifene, a bisphosphonate, calcitonin, tribolone, parathyroid hormone, strontium ranelate, a growth factor, or a cytokine.

5. A method for characterizing a cancer cell comprising determining whether the cancer cell expresses ER-α36.

6. The method of claim 5 wherein the ER-α36 is associated with the cell plasma membrane.

7. The method of claim 5 wherein the ER-α36 is present in the cell cytoplasm.

8. The method of claim 5 wherein the determining comprises use of an antibody that specifically binds to ER-α36 and does not bind to ER-α66, ER-α46, or to ER-β.

ABSTRACT

The invention provides methods and materials that are useful for modulating the expression, activity, or expression and activity of estrogen receptors. The invention also provides methods that can be used to screen for an agents that binds to an estrogen receptor.

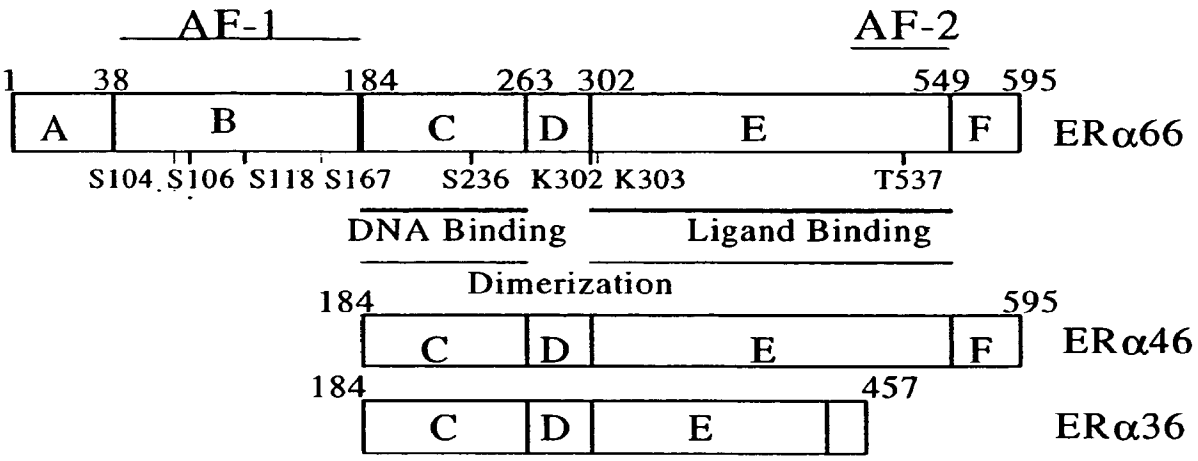

AF-1
AF-2
1
38
184
263
302
549
595
A
B
C
D
E
F
ERα66
S104
S106
S118
S167
S236
K302
K303
T537
DNA Binding
Ligand Binding
Dimerization
184
595
C
D
E
F
ERα46
184
457
C
D
E
ERα36

Fig. 1

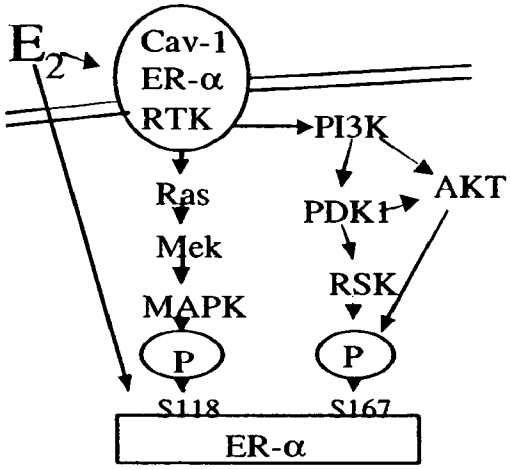
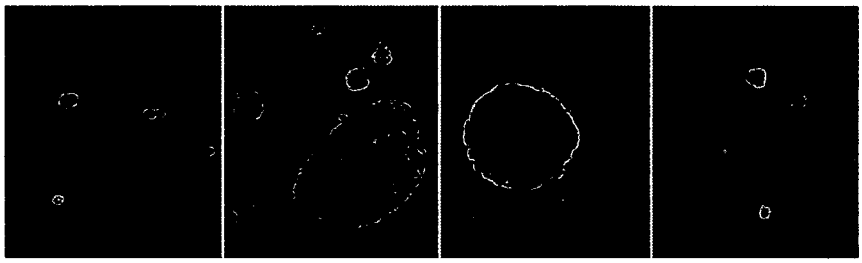


Fig. 2



MCF10A+E2 MCF7 +E2 ST1+E2 ST1-E2

Fig. 3

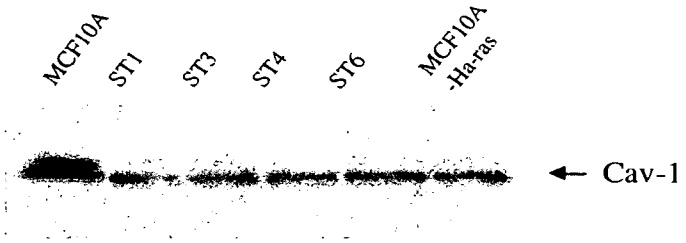


Fig. 4

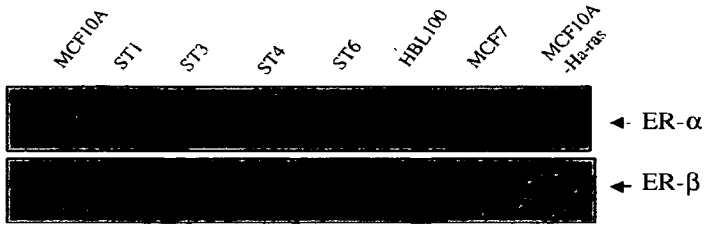


Fig. 5

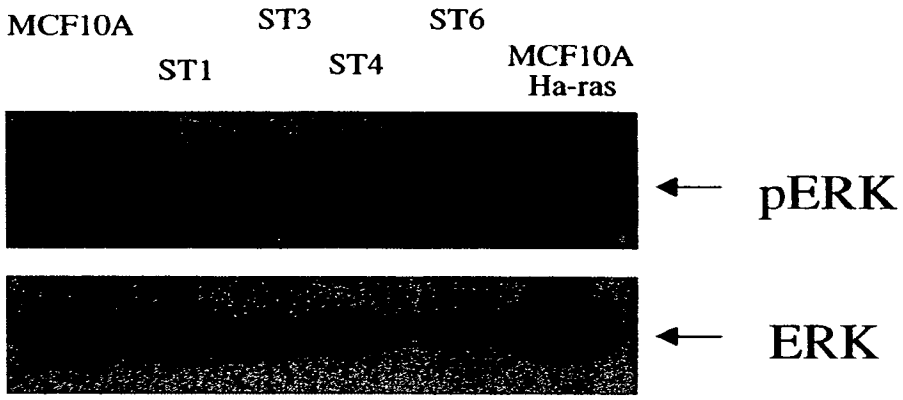


Fig. 6

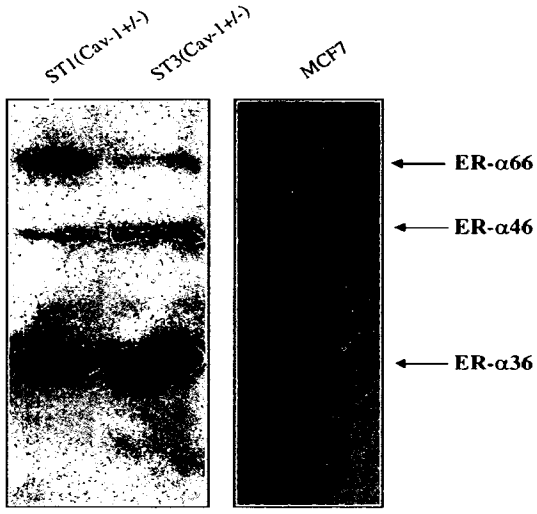


Fig. 7

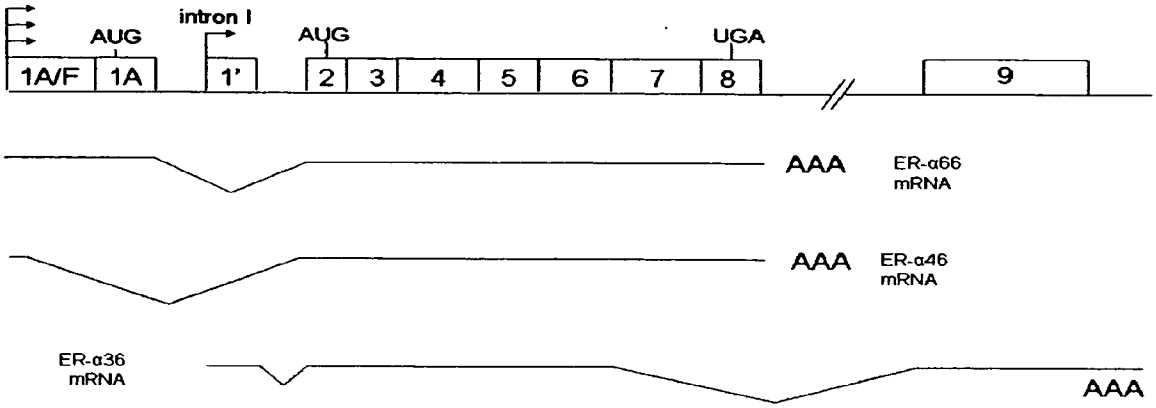

AUG
intron I
AUG
UGA
1A/F
1A
1'
2
3
4
5
6
7
8
9
AAA
ER-α66
mRNA
AAA
ER-α46
mRNA
ER-α36
mRNA
AAA

Fig. 8

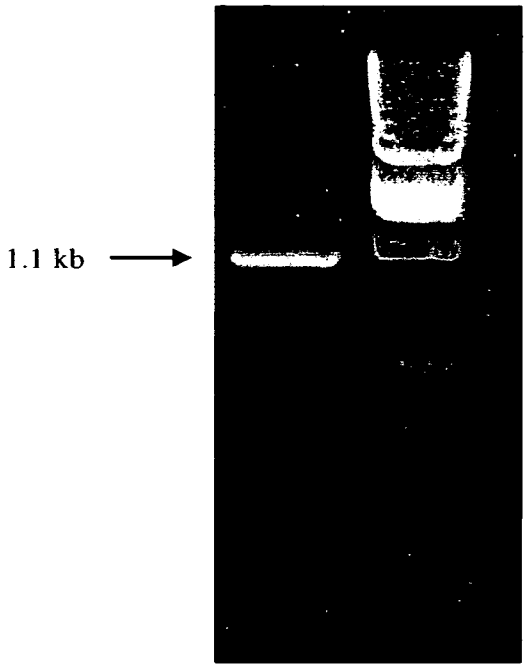


Fig. 9

1 mamesaketr ycavcndyas gyhygvwsce gckaffkrsi qghndymcpa tnqctidknr
61 rkscqacrlr kcyevgmmkg girkdrrggr mikhkrqrdd gegrgevgsa gdmraanlwp
121 splmikrskk nstalsltad qmvsalldae ppilyseydp trpfseasmm gltnladre
181 lvhminwakr vpgfvdltlh dqvhllecaw leilmiglvw rsmehpgkll fapnllldrn
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301 ifgnkwfprv

Fig. 10

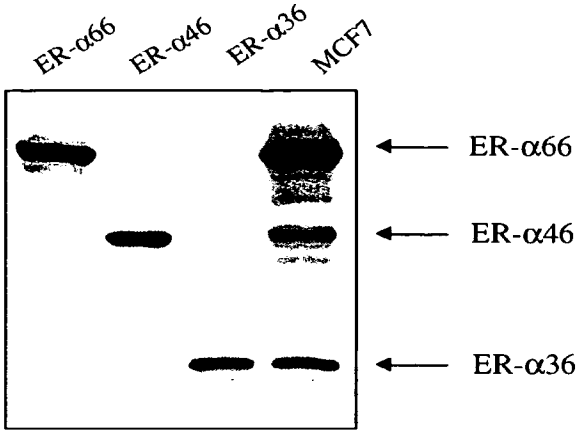

Fig. 11


GGTACCCGCGCCCGCGCCGCCCGTCGGGGTGGCCGCCGCGCCCGGCAGGAGGGAGGGAGGG
Sp1
AP-2
Krox-20
Sp1
AGGGAGGGAGAAGGGAGAGCCTAGGGAGCTGCGGGAGCCGCGGGACGCGCGACCCGAGGGT
Sp1
Sp1
AhR
GCGCGCAGGGAGCCCGGGGCGCGCGGCCCAGCCCGGGGGTTCTGCGTGCAGCCCGCGCTGC
WT1
GTTCAGAGTCAAGTTCTCTCGCCGGGCAGCTGAAAAAAACGTACTCTCCACCCACTTACCGTCCG
YY1
c-Fos
TGCGAGAGGCAGACCCGAAAGCCCGGGCTTCCTAACAAAACACACGTTGGAAAACCAGACAAAG
NF-kappaB
CAGCAGTTATTTGTGGGGGAAAACACCTCCAGGCAAATAAACACGGGGCGCTTTGAGTCACTTG
GR
NF-kappaB
GATA-1
AP-1
c-Jun
AP-1
ER
c-Fos
GGAAGGTCTCGCTCTTGGCATTTAAAGTTGGGGGTGTTTGGAGTTAGCAGAGCTCAGCAGAGTTT
NF-kappa
TATTTATCCTTTTAATGTTTTTGTTTAATGTGCTCCCCAAATTTCCTTTCATCTAGACTATTTGATTG
TBP
GAAATATGTCAGCTATGATGATGACTTTCTGGGAAGCGATTCCTGTCACCCGCTTTCCCCTCCTC
CCCACCCCACGTCCTGGGGCTTTAGAGAGCGATTGGGAGTTGAATGGGTCTGATTTCGGAGTTA
GCTGGCTGAGTCCGCGCTGGAGCGGATTGCTGGCATGTGACTTCTGACAGCCGGAAATTTGTAG
cDNA
GTGTCCCGCGAGTTTAAAACAAGCCATATGGAAGCACAAGTGCTTAAAAA

Fig. 12